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Award Number: W81XWH-06-1-0075

TITLE: SoyCaP: Soy and Prostate Cancer Prevention

PRINCIPAL INVESTIGATOR: Jill M. Hamilton-Reeves, Ph.D.
Mindy S. Kurzer, Ph.D.
Joel Slaton, M.D.

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, Minnesota 55455-2070

REPORT DATE: November 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-11-2006	Annual Summary	24 Oct 2005 – 23 Oct 2006

4. TITLE AND SUBTITLE

SoyCaP: Soy and Prostate Cancer Prevention

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0075

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Jill M. Hamilton-Reeves, Ph.D.; Mindy S. Kurzer, Ph.D. and Joel Slaton, M.D.

E-Mail: jhreeves@umn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota
Minneapolis, Minnesota 55455-2070

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The main objective of this project is to evaluate the effects of soy phytoestrogens on reproductive hormones and prostate tissue markers of cell proliferation and androgen action in men at high risk of prostate cancer. The hypothesis is that alteration of endogenous hormones is a mechanism by which soy phytoestrogens prevent prostate cancer. A randomized parallel arm study is being performed, in which 58 men at high risk of prostate cancer were randomized to receive one of three dietary supplements for six months: 1) soy protein isolate containing isoflavones; 2) isoflavone-poor soy powder; or 3) isoflavone-free milk powder. Urine and blood is collected at 0, 3 and 6 mo, for evaluation of serum hormones and prostate specific antigen, as well as urinary estrogen and phytoestrogen metabolites. At 0 and 12 mo, prostate biopsies aperformed to evaluate prostate tissue expression of apoptosis (Bax, Bcl-2), proliferation (PCNA), and androgen receptor density. We found isoflavone-rich soy protein isolate suppressed androgen receptor density, increased urinary estrogen excretion and increased the 2:16-OH estrone ratio in the urine. We also observed a trend toward a lower rate of prostate cancer in the men in the soy groups compared to the men in the milk group

15. SUBJECT TERMS
Prostate cancer, isoflavones, soy

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	92	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

The low risk of prostate cancer in Asia is thought to be due to dietary factors, including soy consumption. Studies showing an inverse association between prostate cancer risk and urinary excretion of soy phytoestrogens suggest that phytoestrogens contribute to the cancer-preventive effects of soy. One mechanism by which soy phytoestrogens are thought to be cancer-preventive is *via* reduction of endogenous sex hormones known to stimulate prostate cell growth. Despite the interest in soy phytoestrogens for prevention of prostate cancer, there have been no studies in men to evaluate the effects of soy phytoestrogen consumption on sex steroids and prostate tissue biomarkers, and no studies evaluating effects of phytoestrogen metabolism on sex steroids in men.

The main objective of this project is to evaluate the effects of soy phytoestrogen consumption on reproductive hormones and prostate tissue markers of cell proliferation and androgen action in men at high risk of prostate cancer. The underlying hypothesis is that alteration of endogenous hormones is a mechanism by which soy phytoestrogens prevent prostate cancer.

The specific aims of this study (SoyCaP) are to compare the effects of consumption of phytoestrogen-containing soy protein, phytoestrogen-free soy protein, and milk protein, on risk factors for prostate cancer (endogenous hormones, prostate specific antigen, prostate tissue markers of cell proliferation and hormone action), in men at high risk for prostate cancer. Comparing the three groups will enable us to distinguish the specific effects of soy phytoestrogens from effects caused by other soy components. A randomized parallel arm study will be performed, in which 63 men at high risk of prostate cancer will be randomized to receive one of three dietary supplements for six months: 1) soy powder containing 1 mg phytoestrogens/kg body weight; 2) phytoestrogen-free soy powder; and 3) phytoestrogen-free milk powder. Urine and blood will be collected at 0, 3 and 6 months, for evaluation of serum hormones (testosterone, dihydrotestosterone, androstenedione, dehydroepiandrosterone, estradiol, estrone, 3α, 17β-androstanediol glucuronide, sex hormone binding globulin) and prostate specific antigen, as well as urinary estrogen and phytoestrogen metabolites. Before and after the intervention, prostate biopsies will be performed to evaluate prostate tissue expression of apoptosis (Bax and Bcl-2), proliferation (proliferating cell nuclear antigen (PCNA)), epidermal growth factor receptor (EGFr), estrogen receptor beta (ERβ) and androgen receptor (AR)density.

Data from *in vitro*, animal and epidemiological studies suggest that androgens and estrogens play a role in prostate carcinogenesis. Soy isoflavones have been shown to alter sex steroids in women in a potentially beneficial direction, yet such studies in men have not been reported. Studies of the hormonal effects of soy isoflavones in men will contribute to our knowledge of the cancer-preventive mechanisms of soy isoflavones, and may lead to dietary recommendations for prevention of prostate cancer.

BODY

According to the original statement of work, the following tasks were to be performed during the two years of this project:

Task 1: Work with IRB and approval from Army's Office of Research Protection and coordinate with Veteran's Administration to establish all study protocols (months 0-6).

Task 2: Determine the effects of soy phytoestrogen consumption on serum hormones, sex hormone binding globulin (SHBG) and prostate specific antigen (PSA); urinary phytoestrogens and estrogen metabolites; and prostate biopsy biomarkers in men at high risk of prostate cancer.

- Analyze samples from cohort #1 (30 men): serum hormones and SHBG by RIA; serum free and total PSA by ELISA; urine estrogen metabolites and phytoestrogens by GC-MS; biopsy slides by immunohistochemistry (months 6-7)

- Recruit 15 men at high risk of prostate cancer (cohort #2) and randomize into three intervention groups (month 9-12)
- Perform feeding study in cohort #2; process and store serum, urine and biopsy slides (months 9-15)
- Analyze samples from cohort #2: serum hormones and SHBG by RIA; serum free and total PSA by ELISA; urine estrogen metabolites and phytoestrogens by GC-MS; biopsy slides by immunohistochemistry (months 15-17)

- Recruit 15 men at high risk of prostate cancer (cohort #3) and randomize into three intervention groups (month 15-18)
- Perform feeding study in cohort #3; process and store serum, urine and biopsy slides (months 15-22)
- Analyze samples from cohort #3: serum hormones and SHBG by RIA; serum free and total PSA by ELISA; urine estrogen metabolites and phytoestrogens by GC-MS; biopsy slides by immunohistochemistry (months 18-22)

Task 3: Perform data analyses and prepare manuscripts for publication (months 22-24)

All tasks have been completed. Three manuscripts have been prepared for publication and we plan to submit them in the next month. Serum hormone data and steroid hormone expression data are reported in appendix A. Estrogen metabolite data are reported in appendix B. PSA and tissue antigen expression data are reported in appendix C.

Recruitment Summary

The last study year: October 2005 - December 2006:

From October 2005 - December 2006

To date a total of 90 subjects have been enrolled, out of which 56 have completed the study, 10 have dropped out, and 24 consented but never started the study. Data from 2 subjects that completed 3 months of the study with good compliance were analyzed and included in results.

Table 1. Enrollment Summary

	Completed 6 months	Currently completing study	Withdrew after starting	Consented but did not start	Total enrollment
Prior to grant	37	0	9	19	65
10/05–12/06	19	0	1	5	25
TOTAL	56	0	10	24	90

Of the subjects enrolled, 5 individuals (18.5%) did not start the study as a result of inconvenience or placement on a physician monitored weight-loss plan.

One person withdrew after the starting the study. The reason for withdrawal was discomfort with the powder i.e. feeling of being bloated.

KEY RESEARCH ACCOMPLISHMENTS

- Consumption of isoflavone-rich soy protein isolate suppressed androgen receptor expression, increased urinary estrogen excretion, and increased the 2 hydroxyestrogens to 16α hydroxyestrone ratio.
- Consumption of isoflavone-poor soy protein isolate increased serum estradiol and androstenedione concentrations, showed mixed effects on prostate tissue markers, tended to lower androgen receptor density, increased urinary estrogen excretion.
- A lower rate of prostate cancer development was observed in men of both soy groups compared to the milk group.

REPORTABLE OUTCOMES

Jill M. Hamilton-Reeves obtained her PhD in December of 2006.

Jill Hamilton-Reeves has been named one of five finalists in the American Society for Nutrition Clinical Young Investigator Award Competition, for her abstract "Soy Protein Isolate Increases Urinary Estrogens and the Ratio of 2:16a-hydroxyestrone in Men at High Risk of Prostate Cancer." The top five finalists will each receive a $750.00 award and a plaque inscribed with their name and the meeting year. The oral competition will take place at the Experimental Biology meeting in Washington, D.C. in April and the overall winner will be announced at the Nutrition Societies awards ceremony on Sunday evening, April 29, 2007.

Papers to be submitted for publication:

J. M. HAMILTON-REEVES, S. A. REBELLO, W. THOMAS, J. W. SLATON, and M. S. KURZER
Soy protein isolate suppresses androgen receptor expression without altering estrogen receptor beta expression or serum hormonal profiles in men at high risk of prostate cancer. Journal of Nutrition

J. M. HAMILTON-REEVES, S. A. REBELLO, W. THOMAS, J. W. SLATON, and M. S. KURZER. Soy protein isolate increases urinary estrogens and the ratio of 2:16α-hydroxyestrone in men at high risk of prostate cancer Cancer Research

J. M. HAMILTON-REEVES, S. A. REBELLO, W. THOMAS, M. S. KURZER, and J. W. SLATON. Effects of soy protein isolate consumption on prostate cancer biomarkers in men at high risk of prostate cancer: results from the SoyCap trial. The Prostate

Papers published:
Hamilton-Reeves J and Kurzer MS. (2003) Effects of soy isoflavone consumption on reproductive hormones in males. *Soy Connection* 11(4): 3-5.

CONCLUSIONS

The objective of this project was to evaluate the effects of isoflavone-rich soy protein isolate on prostate cancer biomarkers in men at high risk of cancer and to determine whether or not isoflavones are the responsible bioactive components of soy. Isoflavone-rich soy protein isolate suppressed androgen receptor density, increased urinary estrogen excretion, and increased the 2:16 OH-E1 ratio in the urine. Similarly, isoflavone-poor soy protein isolate tended to lower androgen receptor density, and it significantly increased urinary estrogen excretion. Moreover, the isoflavone-poor soy protein isolate increased serum estradiol and androstenedione concentrations, and showed mixed effects on prostate tissue markers. Interestingly, we observed a trend toward a lower rate of prostate cancer development in the men in the soy groups compared to the men in the milk group. Taken together, these findings suggest that soy protein isolate mediates prostate cancer preventive effects in men at high risk of developing prostate cancer. However, it is unclear whether other soy constituents were responsible for the effects, or if the low level of isoflavones in the isoflavone-poor soy protein isolate were sufficient to exert the observed effects.

REFERENCES

None

APPENDICES

Appendix A:

J. M. HAMILTON-REEVES, S. A. REBELLO, W. THOMAS, J. W. SLATON, and M. S. KURZER

Soy protein isolate suppresses androgen receptor expression without altering estrogen receptor beta expression or serum hormonal profiles in men at high risk of prostate cancer. Journal of Nutrition

Appendix B:

J. M. HAMILTON-REEVES, S. A. REBELLO, W. THOMAS, J. W. SLATON, and M. S. KURZER. Soy protein isolate increases urinary estrogens and the ratio of 2:16α-hydroxyestrone in men at high risk of prostate cancer Cancer Research

Appendix C:
J. M. HAMILTON-REEVES, S. A. REBELLO, W. THOMAS, M. S. KURZER, and J. W. SLATON. Effects of soy protein isolate consumption on prostate cancer biomarkers in men at high risk of prostate cancer: results from the SoyCap trial. The Prostate

Appendix A

Soy protein isolate suppresses androgen receptor expression without altering estrogen receptor beta expression or serum hormonal profiles in men at high risk of prostate cancer[1]

J. M. HAMILTON-REEVES[2], S. A. REBELLO[2], W. THOMAS[3], J. W. SLATON[4], and M. S. KURZER[2*]

[1] The Soy and Prostate Cancer Prevention (SoyCaP) trial was supported by grant DAMD 17-02-1-0101 (MSK) and W81XWH-06-1-0075 (JHR) from the United States Army Department of Defense Prostate Cancer Research Program. The protein isolates were donated by The Solae Company, St. Louis, MO. Neither sponsor was involved in writing this report.

[2]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108, USA

[3]Division of Biostatistics in the School of Public Health, University of Minnesota, Minneapolis, MN 55455, USA

[4] Department of Urologic Surgery, University of Minnesota, Minneapolis, MN 55455 and Department of Urology Veterans Administration Medical Center, Minneapolis, MN 55417, USA

[Running title: SOY EFFECTS ON HORMONES IN MEN]

[*] Corresponding author: Dr. Mindy Kurzer, Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, Minnesota 55108, USA, Phone: (612) 624-9789; Fax: (612) 625-5272; E-mail: mkurzer@umn.edu.

ABSTRACT WORD COUNT: 226

MANUSCRIPT WORD COUNT: 4417 (*including section headers*)
- Introduction = 316
- Methods = 955
- Results = 739
- Discussion = 1,029
- Acknowledgements = 32

TABLES: 6

ABSTRACT

The purpose of this study was to determine the effects of soy protein isolate consumption on circulating hormone profiles and hormone receptor expression patterns in men at high-risk for developing prostate cancer. Fifty-eight men were randomly assigned to consume one of three protein isolates containing 40 g protein/d: 1) soy protein isolate (SPI+) (107 mg isoflavones/d); 2) alcohol-washed soy protein isolate (SPI-) (< 6 mg isoflavones/d); or 3) milk protein isolate (MPI) (0 mg isoflavones/d). For six months, the men consumed the protein isolates in divided doses twice daily as a partial meal replacement. Serum samples collected at 0, 3, and 6 months were analyzed for estradiol, estrone, sex hormone binding globulin, androstenedione, androstanediol glucuronide, dehydroepiandrosterone sulfate, dihydrotestosterone, testosterone, and free testosterone concentrations by radioimmunassay. Prostate biopsy samples obtained pre- and post- intervention were analyzed for androgen receptor (AR) and estrogen receptor beta (ERβ) expression by immunohistochemistry. At 6 months, consumption of SPI+ significantly suppressed AR expression but did not alter ERβ expression or circulating hormones. Consumption of SPI- significantly increased estradiol and androstenedione concentrations, and tended to suppress AR expression. Although the effects of SPI- consumption on estradiol and androstenedione are difficult to interpret and the clinical relevance is uncertain, these data show that AR expression in the prostate is suppressed by soy protein isolate consumption, which may be beneficial in preventing prostate cancer.

KEY WORDS: soy, isoflavone, equol, androgen receptor, prostate cancer

INTRODUCTION

Steroid hormones are known to modulate growth of the prostate gland, and elevated levels of androgens have been associated with prostate cancer risk (1, 2). Consumption of soy foods is thought to contribute to prostate cancer prevention as a result of the hormonal properties of soy isoflavones, either through altered endogenous circulating hormones or hormone-receptor signaling. Cell culture studies have suggested that the isoflavonoids, genistein and equol, exert the most significant hormonal effects. Genistein inhibits the activity of 5α-reductase and 17β-hydroxysteroid dehydrogenase, enzymes required for androgen synthesis (3, 4), and alters the expression of androgen-regulated genes in human prostate cancer cell lines (5). The isoflavonoid equol, a bacterially-derived metabolite of the isoflavone daidzein, has been shown to sequester dihydrotestosterone (DHT) from the androgen receptor (AR) in rat prostate tissue (6). The observation that isoflavones accumulate in the prostate gland suggests that they may exert local biological activity (7-10). Together, these mechanistic and distribution studies suggest that isoflavones may mimic or modulate endogenous hormones relevant to prostate carcinogenesis.

Despite evidence from in vitro studies, human intervention studies have reported inconsistent effects of soy or isoflavone consumption on circulating hormone profiles in men. Although statistically significant suppression of total testosterone (11, 12), sex hormone binding globulin (SHBG) (13), DHT (14), dehydroepiandrosterone (15), estrone (16), and free androgen index (14), and increased concentrations of SHBG (17) and DHT (18) have been reported, the majority of the twenty-two intervention studies to date have failed to find statistically significant changes in circulating sex steroid

hormones (11-32). Generally, the studies reporting significant changes were carried out in older men for a relatively long duration. None of the published studies reported equol-excretor status effects on circulating hormone response to soy isoflavone interventions in men.

Circulating hormone profiles may fail to accurately reflect prostate tissue exposure, and evaluating hormone receptor expression patterns in the prostate may provide additional evidence concerning the role of soy as a cancer preventive dietary agent. The androgen receptor (AR) mediates the action of androgens, and AR expression is a potential marker for prostate cancer prognosis (33). Dietary genistein has been shown to down-regulate AR mRNA expression in rodents (34, 35), and genistein has been shown to suppress AR activity through an estrogen receptor β-dependent mechanism in LNCaP cells (36). During cancer progression, the expression of estrogen receptor-alpha (ERα) increases as estrogen receptor-beta (ERβ) decreases (37-39). ERβ upregulation may be protective by counteracting androgen growth stimulation and inducing antioxidant enzymes (37). Animal studies have substantiated the affinity of genistein to ERβ, but have reported that prolonged genistein exposure suppresses ERβ expression (34, 40). Despite these data, there are no studies published to date that have evaluated the effects of soy protein isolate consumption on AR and ERβ expression in men, although one study has reported that an isoflavone extract derived from red clover failed to alter AR expression compared to historically-matched controls (27).

The objective of this project was to evaluate the effects of isoflavone-rich soy protein isolate consumption on circulating concentrations of reproductive hormones and prostate tissue markers of estrogen and androgen receptor expression in men at high

risk of prostate cancer. The effects of an isoflavone-rich soy protein isolate were compared to those of an isoflavone-poor soy protein isolate in order to determine whether the isoflavones are the responsible bioactive constituents. The underlying hypothesis was that isoflavone-rich soy protein isolate consumption would reduce circulating hormones, down-regulate AR expression, and upregulate ERβ expression.

MATERIAL AND METHODS

Subjects. Fifty-eight men, aged 50-85, were recruited at the Minneapolis Veteran's Administration Medical Center Urology Clinic from a group of patients that had already undergone a transrectal ultrasound and biopsy. Patients in this study were either at high risk for developing prostate cancer (*n* = 53), or had low-grade prostate cancer that was being followed by active surveillance (*n* = 5). Subjects were considered high risk if they had high-grade prostatic intraepithelial neoplasia (PIN) (*n* = 50) and/or ASAP (atypical small acinar proliferation) (*n* = 14). The subjects with prostate cancer had Gleason scores of less than 6 and were not receiving any other prostate cancer therapy. Subjects were recruited by urologic physicians, and the research nurse reviewed the patients' medical records to determine that eligibility criteria were met. Exclusionary criteria included BMI > 40 kg/m^2, prostate cancer that required medical treatment, prostatitis, alcohol consumption greater than fourteen drinks/week, soy or milk allergy, regular antibiotic use, or renal insufficiency.

Eighty-seven subjects were screened for the study; 21 chose not to participate after attending the orientation session, and 66 subjects began the study. Eight subjects withdrew from the study before their 3-month appointment [disliked the study treatment powder (*n* = 3), inconvenienced by study demands (*n* = 2), gastrointestinal discomfort (*n* = 1), chose conventional prostate cancer treatment (*n* = 1), weight gain (*n* = 1)]. Three subjects

completed 3 months of the study with good compliance, but chose not to finish due to inconvenience of the study demands, and 55 subjects completed the full 6- month study.

Data from 58 subjects were included in the serum hormone analysis, and 42 subjects were included in the hormone receptor expression analysis. Fewer participants were eligible for the hormone expression analysis because 3 subjects did not undergo the final prostate biopsy [liver cancer diagnosis (*n* = 1), heart condition (*n* = 1), not clinically indicated (*n* = 1)], and 13 subjects had insufficient biopsy tissue at either baseline or post-intervention for the analyses. All 58 subjects who completed the study were Caucasian.

Study design. The University of Minnesota Institutional Review Board: Human Subjects Committee, the Minneapolis Veterans Affairs Institutional Review Board, and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board approved the study protocol and all subjects provided informed consent, attended an orientation session, and were provided with a study handbook. The 6-month intervention study used a randomized, single-blinded, placebo-controlled, parallel design. Free-living subjects supplemented their diets with one of three randomly assigned protein isolates: 1) soy protein isolate high in isoflavones (SPI+); 2) soy protein isolate that had most of the isoflavones removed by alcohol extraction (SPI-); or 3) milk protein isolate (MPI) (The Solae Company; St. Louis, MO). The protein isolates were consumed in divided doses twice daily and contributed 40 g protein/d and 200-400 kcal/d (1 kcal = 4.184 kJ). The isoflavone content of the protein isolates expressed in aglycone equivalents was 107 $\pm$ 5.0 mg/d for the SPI+; < 6 $\pm$ 0.7 mg/d for the SPI-; and 0 mg/d for the MPI (mean $\pm$ SD). The mean distribution of isoflavones was 53% genistein, 35% daidzein, and 11% glycitein in SPI+, and 57% genistein, 20% daidzein,

and 23% glycitein in SPI- as analyzed by Dr. Pat Murphy (Department of Food Science and Human Nutrition, Iowa State University). The packets of protein isolate were numbered and patients were not told which treatment protein isolate they had been assigned until all subjects completed the intervention. Only the study coordinators who administered the protein isolates knew the group to which each participant belonged. Compliance was assessed by counting the number of times the patient consumed the protein isolate as self-reported in recording calendars given to them, and average compliance was 94%. Dietary and herbal supplements were allowed, and participants were asked to avoid changing dosages or adding new supplements to their regimen during the study. Subjects consumed their habitual diets, and received detailed instructions to exclude soy products in order to minimize isoflavone consumption from other sources.

Serum collection and analysis. At baseline, subject height and weight were measured in street clothing without shoes. Fasting blood was collected in the morning at 0, 3, and 6 months. Serum was separated and aliquots were frozen at –70°C until analysis. All serum samples were analyzed for testosterone, free testosterone, DHT, androstanediol glucuronide (3α-AG), androstenedione, dehydroepiandrosterone sulfate (DHEA-S), SHBG, estradiol, and estrone. Steroid hormones were analyzed in duplicate by radioimmunoassay, and SHBG was analyzed by immunoradiometric assay (Diagnostics Systems Laboratories, Inc., Webster, TX). Hormone analyses were performed in three batches and all assays required ^{125}I-labeled analyte. Intra-assay variabilities were 3.7% for testosterone, 4.4% for free testosterone, 6.1% for DHT, 4.5% for 3α-AG, 4.4% for androstenedione, 2.3% for DHEAS, 4.4% for SHBG, 3.9% for estradiol, and 4.3% for

estrone. An internal control was utilized to determine variability among batches, and inter-assay variabilities were between 9% and 30% for all analytes. All three serum samples for each participant were analyzed in the same batch.

Urine collection and analysis. To assess equol-producer status, 24-hr urine was collected in plastic containers containing one gram of ascorbic acid per liter and separated into aliquots after the addition of sodium azide to a final concentration of 0.1%. Aliquots were frozen at –20°C until analysis. Equol was determined by high performance liquid chromatography and mass spectrometry as previously described (41). The intra-assay coefficient of variation for equol was 8.2%, and inter-assay coefficient of variation was 12.5%.

Dietary intake and analysis. Food records were completed for 3 days before each clinic visit. A Registered Dietitian taught study participants how to keep accurate food records. Patients were encouraged to use household scales and volumetric tools and to submit food labels from unusual foods. Study coordinators reviewed each food record for completeness and clarified any ambiguities with the participant at each clinic visit. Food records were analyzed with Nutritionist V (Version 2.3) (42) and for each 3-day food record mean intakes of energy, macronutrients, saturated fat, cholesterol, fiber, vitamin D, vitamin E, calcium, selenium, and zinc were calculated.

Tissue collection and analysis. Transrectal ultrasound and 12 prostate core biopsies were performed before the initial screening and performed again at the 6 month clinical visit. Biopsy cores were preserved in formalin for 24 hours and then embedded in paraffin blocks. Once the specimens were processed, the paraffin-embedded blocks were sectioned onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). The

histological diagnoses were determined during a routine pathological evaluation. The sections analyzed primarily represented normal, hyperplastic, or preneoplastic glands. Immunohistochemistry was performed to assess AR and ERβ expression on all biopsy specimens collected from eligible study participants. The tissue sections were deparaffinized in AmeriClear (Scientific Products**,** Stockton, CA), rehydrated in graded alcohol, and transferred to phosphate buffered saline (PBS) (pH 7.3). Antigen retrieval was achieved by pressure-cooking tissue sections at 15 psi in citrate buffer with a pH of 6.0 for 10 minutes. Sections were treated in quenching solution (3% H_2O_2 in 100% MeOH) for 5 minutes. The samples were washed and incubated with a protein-blocking solution for 20 minutes (10% milk, 5% serum, and 1% bovine serum albumin). They were then incubated overnight at 4° C with rabbit polyclonal anti-ERβ antibody (ab3577; Abcam Inc., Cambridge, MA; 1:1000) for the ERβ assay, or incubated at room temperature for 30 minutes with the mouse monoclonal anti-AR antibody (AM256-2M; BioGenex, San Ramon, CA; RTU) for the AR assays. Next, samples were rinsed and incubated with the appropriate biotinylated secondary antibody, followed by Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Color reaction was developed using diaminobenzidine (DAB) as the chromagen. The reactions were stopped with water and counterstained with Harris' Modified Hematoxylin (Fisher Scientific, Pittsburgh, PA), dehydrated, cleared, and mounted. Appropriate positive and negative controls were included in all staining runs.

The stained slides were photographed using an Olympus microscope (BX60) linked to a camera (Diagnostics Instruments Inc.) and computer. The digital images were optimized for quantification using Adobe Photoshop, Version 7.0 (San Jose, CA).

Disrupted glands and glands on the edge of tissue sections were excluded from analysis to avoid false positives. Positive staining was assessed without prior knowledge of histological grading by a technician using the HSCORE system as previously described (43). The range of the HSCORE is a minimum of 1 and a maximum of 4 (1 indicated absent staining; 4 indicated intense staining). Both the intensity of immunostaining and the percentage of immunopositive areas were recorded at 40X magnification. To enhance scorer objectivity, brightness and contrast levels of the digital images were scaled up in order to identify lightest staining and scaled down to identify the most intense staining. The sum of the product of percent positive areas and intensity gave the final score for the fields. A mean of 6 intact glands (range: 2 – 15) per slide for ERβ and a mean of 8 intact glands (range: 3 – 19) per slide for AR were averaged to derive the HSCORE.

Excluded from analysis. The following data were excluded from statistical analysis: 6 month dietary intake from one participant reporting unusually low consumption (mean < 500 kcal) (1 kcal = 4.184 kJ) during the 3-day food diary as a result of illness; 3 month DHEA-S that was above normal range (16,400 nmol/L) and inconsistent with the participant's baseline and 6 month measurements; all DHEA-S measurements from one participant with abnormally high 3 and 6 month DHEA-S concentrations (9,005 and 10,078 nmol/L respectively) compared to baseline; and all SHBG measurements from one subject with undetectable SHBG in the serum (< 3 nmol/L). One subject did not consume the treatment powder for 3 days prior to his 6 month appointment as a result of illness, so he was excluded from the 6 month equol excretion analysis.

Statistical analysis. The data appeared normally distributed and had similar variance among groups. Analysis of covariance was used to compare group means adjusted by their baseline values, and for androstenedione, treatment by baseline interaction (Proc GLM, SAS Institute Inc., *SAS® 9.1*, Cary, NC: SAS Institute Inc., 2003). In addition, we carried out pre-planned pairwise comparisons as dictated by the study hypotheses: 1) SPI+ would differ from MPI and 2) SPI+ would differ from SPI-. Paired t-tests were used to test for significant within-group changes over time. In addition, these covariates were screened as adjusters: baseline body weight, equol excretor status, and energy and nutrient intake. Paired t-tests were used to compare baseline characteristics of equol excretors versus non-excretors. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline. Baseline anthropometrics, cancer status, and dietary intake did not differ among the groups **(TABLE 1)**, except that the MPI group had a higher body weight and the SPI- group consumed significantly higher protein, calcium, and zinc at baseline **(TABLE 2)**. There were no differences in baseline serum hormone and SHBG concentrations or steroid receptor expression patterns among the groups **(TABLES 3 & 4)**.

Anthropometrics and dietary intake. Weight did not change from baseline to 3 or 6 months in any group **(TABLE 2)**, and the significant differences in body weight seen at baseline were maintained. Protein, calcium, and vitamin D intakes increased in all groups during the study as a result of their concentrations in the protein isolates, and the differences in protein, calcium, and zinc intake at baseline disappeared at 3 and 6 months. At 3 months, total and saturated fat consumption were reduced in the SPI-

group. During the study, energy, carbohydrate, cholesterol, fiber, vitamin E, selenium, and zinc intakes did not change for any group. Dietary and herbal supplement usage did not differ between groups (data not shown). Weight and protein intake differences between groups were unrelated to altered hormone concentrations or steroid receptor expression patterns.

Steroid Receptors. AR expression was lower in prostate biopsies after 6 months in the SPI+ group compared to the MPI group (pairwise comparison, $P = 0.04$) and approached a significant difference in the SPI- group compared to the MPI group (pairwise comparison, $P = 0.09$). In contrast, there were no differences in ERβ expression among the groups (TABLE 3).

Estrogens. During the study, estradiol was significantly increased in the SPI- group at 3 and 6 months, and by 6 months, baseline-adjusted estradiol concentrations were significantly higher in the SPI- group compared to the other two groups (TABLE 4). Estrone was also significantly increased in the SPI- group at 3 and 6 months, and estrone concentrations were significantly higher than the MPI group at 3 months but not significantly different at 6 months.

Androgens and SHBG. Androstenedione concentrations were significantly higher in the SPI+ group than the MPI group at 3 months. At 6 months, androstenedione was significantly increased in the SPI- group and resulted in significantly higher androstenedione concentrations than the SPI+ group (TABLE 4). At both 3 and 6 months, DHEAS was higher in the SPI- group than the other two groups, and at 3 months, 3α-AG was higher in the SPI- group than the other two groups. At 3 months, DHT concentrations were decreased from baseline in the SPI- group. Serum SHBG

concentrations were decreased significantly from baseline at 3 and 6 months in all treatment groups.

Equol-excretor status and hormone profiles. Equol excretor status was only determined in the SPI+ group, because only they consumed sufficient daidzein to excrete equol. At 3 months, there were 5 excretors and 14 non-excretors. Only the 3 month serum hormone data are shown, because just 2 excretors remained at 6 months [dropped out after 3 months (n = 1), excluded data (n = 1), apparently changed excretor status (n = 1)]. Baseline characteristics between excretors and non-excretors were not different, although non-excretors tended to have a higher rate of PIN (P = 0.06) and fewer prostate cancer cases (P = 0.06) (TABLE 5). Estrone concentrations tended to be higher at 3 months in excretors than non-excretors (P = 0.07) (TABLE 6).

DISCUSSION

The present study evaluated men at high risk of prostate cancer to determine the effects of soy protein consumption on serum hormones and prostate tissue steroid receptor expression levels. The major finding was lower AR expression levels and no differences in ERβ expression or circulating hormones in men consuming SPI+ compared to those consuming MPI.

Lower tissue AR expression in the SPI+ group is consistent with research in which dietary phytoestrogens down-regulated AR mRNA expression in adult male rats (34, 35, 44). Our data differ, however, from those of Jarred et al, who reported no differences in AR expression patterns between radical prostatectomy patients treated with isoflavones and historically matched controls (27). The inconsistent results between our study and that of

Jarred et al can be explained by several methodological differences. In the study by Jarred et al, the subjects, who consumed 160 mg isoflavones/d in extracts derived from red clover, were men with advanced prostatic neoplasms treated for short and varied time periods (7-54 d). The tissue sections studied from the radical prostatectomies taken from treated subjects represented cancerous glandular acinae and were compared to sections of cancers from historically matched controls. Our subjects consumed 107 mg isoflavones/d in isoflavone-rich SPI, were earlier in the carcinogenesis continuum, were treated for 6 months each, and all biological samples were evaluated within the same subject before and after the intervention. Furthermore, the gland acinae studied presented either benign, hyperplastic, or preneoplastic tissue.

Consumption of SPI+ did not affect ERβ expression or circulating hormones. The ERβ expression results are inconsistent with studies in animals in which prolonged isoflavone exposure decreased ERβ expression (34, 40). Our hormone results, however, are consistent with most published reports from the clinical setting. The testosterone results are consistent with numerous soy or isoflavone intervention studies in which no change in total testosterone was observed (13-32), but differ from two studies of short duration (11, 12). Our finding of no effect on directly measured free testosterone is similar to all published soy or isoflavone intervention studies to date (12, 15, 16, 21, 23, 25), and our finding of no effect on circulating DHT is consistent with most reports (11, 15, 17, 20-22, 24, 31), although it differs from results of two studies (14, 18), one of which used red clover extract (18). The lack of effect on circulating estradiol or estrone is consistent with the literature (11, 12, 16, 17, 20, 23, 30, 31), although there is one report of decreased estrone in men consuming soymilk for 8-weeks (16).

SHBG was decreased significantly from baseline in all study groups. The finding that consumption of SPI+ decreased SHBG is similar to a report by Mackey et al (13); however, Mackey et al did not observe a significant decrease in SHBG with an isoflavone-poor protein isolate as we did. In contrast to our findings, Habito et al reported increased SHBG in men consuming 35 g of tofu daily for 2 weeks (17), and others have reported no statistically significant changes of SHBG with isoflavone-rich foods or extracts (14, 16, 18, 21-24, 31). Since high protein intake has been associated with decreased SHBG (45), it is likely that the decrease in SHBG from baseline observed in all groups in our study resulted from the subjects' significantly increased protein intake during the study (46).

The hormonal effects observed in the SPI- group were unexpected. Although AR expression was not significantly lower in the SPI- group, AR expression appeared to be intermediate between that of SPI+ and MPI groups. In addition, serum estradiol was increased in the SPI- group and estrone concentrations tended to increase compared to the other two groups. These results are similar to a study in young men by Dillingham et al in which a low-isoflavone protein isolate containing <2 mg isoflavones/d significantly increased estradiol and estrone compared to a milk protein isolate after a 8-week intervention (21). Our results differ, however, from a study in older men by Goldin et al in which a low-isoflavone soy protein isolate containing <2 mg isoflavones/d did not change estradiol or estrone concentrations after a 6-week intervention (20). Interestingly, we found serum estradiol was significantly higher in the SPI- group than in the SPI+ group, whereas in Dillingham et al's study, estradiol in the low-isoflavone group was not significantly different from the high-isoflavone group (21).

Serum androstenedione and DHEAS concentrations were increased in the SPI- group compared to both SPI+ and MPI groups. No other soy protein or isoflavone intervention study has reported a change in circulating androstenedione (13, 18, 20, 21, 31), but all other studies to date have intervened for a shorter duration. Higher DHEAS is consistent with other low-isoflavone soy protein isolate interventions (20, 21). Although DHEAS and androstenedione can be converted by 17β-hydroxysteroid dehydrogenase to testosterone, no significant changes were observed in circulating testosterone, free testosterone, or DHT. Instead, our study population had low, but normal, testosterone concentrations throughout the study. Although DHEAS and androstenedione concentrations have been associated with aggressive prostate cancer (47), our finding of unchanged testosterone and a trend toward lower AR expression suggest neutral effects of SPI- consumption. In fact, since DHEAS and androstenedione may be converted to estradiol and estrone in the prostate gland (48), the increase in DHEAS and androstenedione may have contributed to the observed increase in circulating estradiol and estrone. The hormonal effects of SPI- consumption are likely due to the effects of the alcohol extraction process on SPI constituents.

In conclusion, we found that consumption of isoflavone-rich soy protein for 6 months lowered AR expression levels in the prostate, but did not change ERβ expression or circulating hormones in men at high risk of prostate cancer. Although consumption of the alcohol-extracted soy protein did not significantly lower AR expression, its effect appeared to be intermediate between that of SPI+ and MPI consumption, suggesting that the isoflavones alone may not be responsible for the AR expression decrease, or, alternatively, that the low level of isoflavones in SPI- were

sufficient to alter the AR. Unexpectedly, consumption of SPI-, but not SPI+, significantly increased estradiol and androstenedione concentrations. None of these results were influenced by equol excretion status. These data suggest that consumption of soy protein isolate, regardless of isoflavone content, affects endogenous hormones and receptor expression which may mediate prostate cancer preventive effects.

ACKNOWLEDGEMENTS

This research was funded by the United States Army Department of Defense Prostate Cancer Research Program under awards DAMD 17-02-1-0101 (MSK) and W81XWH-06-1-0075 (JHR). Views and opinions of, and endorsements by the author do not reflect those of the US Army or the Department of Defense. The protein isolates were generously donated by The Solae Company, St. Louis, MO. Immunohistochemistry was performed by Kenji Takamura. The authors would like to thank Kayla Vettling and Ellie Wiener for tissue scoring, and Lori Sorensen, Nicole Nelson, and Mary McMullen for assistance with clinic visits and data entry. We are also quite grateful to the study participants for their time and dedication.

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TABLE 1: Baseline characteristics of subjects [1]

	SPI+	SPI-	MPI
	n = 20	n = 20	n = 18
Age (y)	68 ± 8	68 ± 5	68 ± 7
Body wt (kg)	91 ± 16 *ab*	88 ± 12 *a*	98 ± 15 *b*
Height (cm)	175 ± 7	173 ± 8	176 ± 8
BMI (kg/m^2)	30 ± 5	29± 4	32 ± 6
Prostate Cancer Markers [2]			
PIN (*n* (%))	18 (90)	18 (90)	14 (78)
ASAP (*n* (%))	3 (15)	7 (35)	4 (22)
CaP (*n* (%))	2 (10)	1 (5)	2 (12)

[1] All values are means ± SD except prostate cancer markers which are n (%).

[2] Prostate cancer markers PIN, ASAP, and CaP are not mutually exclusive.

[ab] Means in a row without a common letter differ ($p < 0.05$).

Abbreviations: SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI = milk protein isolate (40 g milk protein); PIN = prostatic intraepithelial neoplasia; ASAP = atypical small acini suspicious for prostatic adenocarcinoma; CaP = prostate cancer

TABLE 2: Anthropometrics and dietary intake [1]

	SPI+ *n* = 20 [2]	**SPI-** *n* = 20	**MPI** *n* = 18
Weight (kg)			
Baseline	91 ± 16 *ab*	88 ± 12 *a*	98 ± 15 *b*
3 Mo	91 ± 16 *ab*	87 ± 12 *a*	98 ± 15 *b*
6 Mo	90 ± 16 *ab*	87 ± 13 *a*	99 ± 15 *b*
Height (cm)			
Baseline	175 ± 16	173 ± 8	176 ± 8
BMI (kg/m^2)			
Baseline	30 ± 5	29 ± 4	32 ± 6
3 Mo	30 ± 5	29 ± 4	32 ± 6
6 Mo	30 ± 5	29 ± 4	32 ± 6
Energy Intake (kcal/d) [3]			
Baseline	2140 ± 620	2260 ± 660	2070 ± 520
3 Mo	2220 ± 720	2030 ± 390	2180 ± 510
6 Mo	2240 ± 410	2120 ± 670	2330 ± 410
Protein (g /d)			
Baseline	83 ± 21 *a*	100 ± 24 *b*	81 ± 25 *a*
3 Mo	* 118 ± 24	* 117 ± 16	* 121 ± 30
6 Mo	* 118 ± 21	* 124 ± 29	* 120 ± 18

Carbohydrate (g/d)			
Baseline	256 ± 106	262 ± 118	236 ± 59
3 Mo	246 ± 97	230 ± 82	232 ± 75
6 Mo	251 ± 61	232 ± 89	256 ± 68
Total Fat (g/d)			
Baseline	86 ± 33	93 ± 32	88 ± 24
3 Mo	80 ± 39	* 74 ± 18	73 ± 30
6 Mo	83 ± 34	80 ± 34	89 ± 26
Saturated Fat (g/d)			
Baseline	27 ± 11	34 ± 14	28 ± 11
3 Mo	27 ± 13	* 26 ± 7	24 ± 12
6 Mo	26 ± 10	29 ± 14	30 ± 10
Cholesterol (mg/d)			
Baseline	324 ± 202	382 ± 153	301 ± 163
3 Mo	307 ± 131	296 ± 115	312 ± 233
6 Mo	328 ± 147	348 ± 175	329 ± 234
Fiber (g/d)			
Baseline	17 ± 9	18 ± 7	16 ± 5
3 Mo	16 ± 8	17 ± 8	15 ± 7
6 Mo	15 ± 9	16 ± 9	15 ± 5

Vitamin D (μg/d)			
Baseline	4 ± 3	4 ± 5	4 ± 3
3 Mo	* 9 ± 4	* 8 ± 3	* 8 ± 2
6 Mo	* 8 ± 2	* 8 ± 3	* 9 ± 2
Vitamin E (mg/d)			
Baseline	8 ± 7	8 ± 5	6 ± 4
3 Mo	6 ± 4	7 ± 10	6 ± 3
6 Mo	7 ± 7	6 ± 3	6 ± 3
Calcium (mg/d)			
Baseline	890 ± 400 *ab*	1230 ± 970 *b*	760 ± 360 *a*
3 Mo	* 2260 ± 440	* 2120 ± 350	* 2200 ± 380
6 Mo	* 2180 ± 290	* 2340 ± 840	* 2190 ± 340
Selenium (mg/d)			
Baseline	0.08 ± 0.05	0.09 ± 0.03	0.08 ± 0.05
3 Mo	0.08 ± 0.03	* 0.06 ± 0.03	0.10 ± 0.11
6 Mo	0.07 ± 0.03	* 0.07 ± 0.02	0.44 ± 1.6
Zinc (mg/d)			
Baseline	10 ± 6 *a*	14 ± 5 *b*	10 ± 5 *a*
3 Mo	11 ± 4	10 ± 8	10 ± 3
6 Mo	9 ± 3	10 ± 5	9 ± 3

[1] All values are means ± SD.

[2] Sample sizes listed at column headings are for all time points except the following: 3 mo, MPI (n = 17), and 6 mo, SPI+ (n = 18) and SPI- (n = 18).

[3] 1 kcal = 4.184 kJ

[ab] Means in a row without a common letter differ ($P < 0.05$).

*Significant within-group change from baseline ($P < 0.05$).

Abbreviations: SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI = milk protein isolate (40 g milk protein)

TABLE 3: Steroid receptor expression (HSCORE) [1]

	SPI+	SPI-	MPI
Androgen Receptor (AR)			
Baseline	1.37 ± 0.06	1.28 ± 0.06	1.23 ± 0.06
6 Mo	1.26 ± 0.05 *a*	1.30 ± 0.05 *ab*	* 1.42 ± 0.05 *b*
Estrogen Receptor β (ERβ)			
Baseline	1.22 ± 0.06	1.32 ± 0.06	1.23 ± 0.06
6 Mo	1.16 ± 0.06	1.18 ± 0.06	1.26 ± 0.05

[1] Baseline data are unadjusted means ± SEM. All other data are least-squares means adjusted for baseline measurement ± SEM. The number of patients evaluated for AR expression was 14 for SPI+, 16 for SPI-, and 14 for MPI. The number of patients evaluated for ERβ expression was 14 for SPI+, 14 for SPI-, and 15 for MPI.

[ab] Means in a row without a common letter differ ($P < 0.05$).

*Significant within-group change from baseline ($P < 0.05$).

Abbreviations: SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI = milk protein isolate (40 g milk protein)

TABLE 4: Serum hormones and SHBG [1]

	SPI+ *n* = 20 [2]	**SPI-** *n* = 20	**MPI** *n* = 18
Estradiol (pmol/L)			
Baseline	67 ± 4	66 ± 4	69 ± 3
3 Mo	75 ± 5	* 76 ± 5	* 62 ± 6
6 Mo	69 ± 3 *a*	* 79 ± 3 *b*	66 ± 3 *a*
Estrone (pmol/L)			
Baseline	157 ± 15	141 ± 10	158 ± 8
3 Mo	150 ± 8 *ab*	* 170 ± 8 *b*	146 ± 8 *a*
6 Mo	152 ± 10	* 171 ± 10	150 ± 10
Androstenedione (nmol/L)			
Baseline	2.9 ± 0.3	2.9 ± 0.3	2.5 ± 0.2
3 Mo	3.0 ± 0.2 *a*	3.0 ± 0.2 *ab*	2.8 ± 0.2 *b*
6 Mo	2.6 ± 0.2 *a*	* 3.4 ± 0.2 *b*	2.9 ± 0.2 *ab*
Androstanediol Glucuronide (nmol/L)			
Baseline	19 ± 3	18 ± 5	16 ± 2
3 Mo	17 ± 2 *a*	24 ± 2 *b*	17 ± 2 *a*
6 Mo	16 ± 2	20 ± 2	18 ± 2
DHEAS (nmol/L) [†]			
Baseline	2202 ± 390	2052 ± 300	1977 ± 370
3 Mo	2040 ± 103 *a*	2715 ± 103 *b*	2126 ± 103 *a*
6 Mo	1937± 154 *a*	2372 ± 146 *b*	1946 ± 150 *a*

DHT (pmol/L)			
Baseline	1547 ± 190	1354 ± 170	1072 ± 110
3 Mo	1242 ± 81	* 1076 ± 79	1119 ± 100
6 Mo	1215 ± 94	1174 ± 89	1229 ± 105
Testosterone (nmol/L)			
Baseline	12 ± 1	13 ± 1	12 ± 1
3 Mo	13 ± 0.5	13 ± 0.6	11 ± 0.6
6 Mo	13 ± 0.6	13 ± 0.5	12 ± 0.6
Free Testosterone (pmol/L)			
Baseline	33 ± 3	34 ± 2	29 ± 2
3 Mo	33 ± 1	33 ± 1	32 ± 1
6 Mo	32 ± 1	32 ± 1	31 ± 1
SHBG (nmol/L) [‡]			
Baseline	63 ± 7	64 ± 8	69 ± 9
3 Mo	* 56 ± 3	* 56 ± 2	* 56 ± 3
6 Mo	* 54 ± 3	* 61 ± 3	* 58 ± 3

[1] Baseline data are unadjusted means ± SEM. All other data are least-squares means adjusted for baseline measurement ± SEM, except androstenedione which is additionally adjusted for interaction between treatment and baseline.

[2] Sample sizes listed at column headings are for all time points except: 3 mo MPI (n = 17), and 6 mo SPI+ (n = 18) and SPI- (n = 19).

[†] Sample sizes differed from other hormones due to excluded data. At 3 mo, SPI+ (n = 19) and SPI- (n = 19). At 6 mo, SPI+ (n = 17) and SPI- (n = 19).

[‡] Sample sizes differed from other hormones due to excluded data. At 3 mo, SPI+ (n = 19), and at 6 mo, SPI+ (n = 18).

[ab] Means in a row without a common letter differ ($P < 0.05$).

*Significant within-group change from baseline ($P < 0.05$).

Abbreviations: SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg/d); MPI = milk protein isolate (40 g milk protein); DHT = dihydrotestosterone; DHEAS = dehydroepiandrosterone sulfate; SHBG = sex hormone-binding globulin

TABLE 5: Baseline characteristics of equol excretors and non-excretors [1]

	Excretors n = 5	Non-excretors n = 14
Age (y)	71 ± 5	67 ± 2
Body wt (kg)	89 ± 10	91 ± 4
Height (cm)	178 ± 2	173 ± 2
BMI (kg/m^2)	28 ± 5	30 ± 1
Prostate Cancer Markers [2]		
PIN (*n* (%))	3 (60)	14 (100)
ASAP (*n* (%))	1 (20)	2 (14)
CaP (*n* (%))	2 (40)	0 (0)
Dietary Intake		
Energy intake (kcal/d) [3]	1828 ± 330	2181 ± 140
Protein (g/d)	70 ± 5	83 ± 4
Carbohydrate (g/d)	206 ± 42	271 ± 30
Fat (g/d)	79 ± 18	84 ± 8
Dietary fiber (g/d)	13 ± 3	19 ± 3

[1] All values are means ± SD or n (%).

[2] Prostate cancer markers PIN, ASAP, and CaP are not mutually exclusive.

[3] 1 kcal = 4.184 kJ

Abbreviations: PIN = prostatic intraepithelial neoplasia; ASAP = atypical small acini suspicious for prostatic adenocarcinoma; CaP = prostate cancer

TABLE 6: Serum hormone concentrations between equol excretors and non-excretors [1]

		Excretors *n = 5*	Non-excretors *n = 14*
Estradiol (pmol/L)			
	Baseline	69 ± 5	68 ± 5
	3 Mo	93 ± 17	70 ± 10
Estrone (pmol/L)			
	Baseline	152 ± 36	159 ± 18
	3 Mo	170 ± 13	147 ± 8
SHBG (nmol/L)			
	Baseline	75 ± 14	62 ± 8
	3 Mo	* 58 ± 7	* 57 ± 3
Androstenedione (nmol/L)			
	Baseline	2.1 ± 0.4	3.2 ± 0.4
	3 Mo	2.8 ± 0.3	3.1 ± 0.2
Androstanediol Glucuronide (nmol/L)			
	Baseline	13 ± 4	21 ± 5
	3 Mo	18 ± 3	18 ± 5
DHEAS (nmol/L) †			
	Baseline	1321 ± 293	2478 ± 524
	3 MO	1968 ± 184	2102 ± 112

DHT (pmol/L)			
	Baseline	1873 ± 631	1428 ± 162
	3 Mo	1270 ± 195	1534 ± 115
Testosterone (nmol/L)			
	Baseline	11 ± 3	13 ± 0.9
	3 Mo	12 ± 0.9	13 ± 0.5
Free Testosterone (pmol/L)			
	Baseline	32 ± 8	33 ± 3
	3 Mo	34 ± 2	34 ± 1

[1] Baseline data are unadjusted means ± SEM. Data from 3 months are least-squares means adjusted for baseline measurement ± SEM, except androstenedione which is additionally adjusted for interaction between treatment and baseline.

[ab] Means in a row without a common letter differ ($P < 0.05$).

* Significant within-group change from baseline ($P < 0.05$).

[†] Non-excretors: 3 Mo ($n = 13$)

Abbreviations: DHT = dihydrotestosterone; DHEAS = dehydroepiandrosterone sulfate; SHBG = sex hormone-binding globulin

Appendix B

Soy protein isolate increases urinary estrogens and the ratio of 2:16α-hydroxyestrone in men at high risk of prostate cancer[†]

J. M. HAMILTON-REEVES[1], S. A. REBELLO[1], W. THOMAS[2], J. W. SLATON[3], and M. S. KURZER[1*]

[1]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108, USA

[2]Division of Biostatistics in the School of Public Health, University of Minnesota, Minneapolis, MN 55455, USA

[3]Department of Urologic Surgery, University of Minnesota, Minneapolis, MN 55455 and Department of Urology Veterans Administration Medical Center, Minneapolis, MN 55417, USA

[Running title: "SOY AND ESTROGEN METABOLITES IN MEN"]

KEY WORDS: soy, isoflavones, estrogen metabolism, 2:16 OH-E1, prostate cancer

ABSTRACT WORD COUNT: 250

MANUSCRIPT WORD COUNT: 2809 (*including section headers*)

[†] The Soy and Prostate Cancer Prevention (SoyCaP) trial was supported by grant DAMD 17-02-1-0101 (MSK) and W81XWH-06-1-0075 (JHR) from the United States Army Department of Defense Prostate Cancer Research Program. The protein isolates were donated by The Solae Company, St. Louis, MO. Neither sponsor was involved in writing this report.

[*] Corresponding author: Dr. Mindy Kurzer, Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, Minnesota 55108, USA, Phone: (612) 624-9789; Fax: (612) 625-5272; E-mail: mkurzer@umn.edu.

ABSTRACT

Specific estrogen metabolites may initiate and promote hormone-related cancers. In epidemiological studies, significantly lower excretion of urinary estradiol (E2) and lower ratio of urinary 2-hydroxy estrogens to 16α-hydroxyestrone (2:16 OH-E1) have been reported in prostate cancer cases compared to controls. Although soy supplementation has been shown to increase the ratio 2:16 OH-E1 in women, no studies have investigated the effects of soy supplementation on estrogen metabolism in men. The objective of this randomized controlled trial was to determine the effects of soy protein isolate consumption on estrogen metabolism in men at high-risk for developing advanced prostate cancer. Fifty-eight men supplemented their habitual diets with one of three protein isolates: 1) soy protein isolate (SPI+) (107 mg isoflavones/d); 2) alcohol-washed soy protein isolate (SPI-) (< 6 mg isoflavones/d); or 3) milk protein isolate (MPI), each providing 40 g protein/d. At 0, 3, and 6 months of supplementation, the urinary estrogen metabolite profile was measured by gas chromatography-mass spectrometry. Both soy groups had higher E2 excretion than the MPI group at 3 and 6 months. After 6 months of supplementation, the SPI+ group had a significantly higher urinary 2:16 OH-E1 ratio than the MPI group. These results were not influenced by equol-excretor status. Increased urinary E2 excretion and 2:16 OH-E1 ratio in men consuming soy protein isolate are consistent with studies in postmenopausal women and suggest that soy consumption may be beneficial in men at high risk of progressing to advanced prostate cancer as a result of effects on endogenous estrogen metabolism.

INTRODUCTION

Prostate cancer development is associated with andropause, when the ratio of circulating estrogens to androgens may increase by up to 40% (1). Increased estrogens are known to suppress testosterone production and compete with androgens for the androgen receptor. It has also been hypothesized that rising estrogen concentrations may cause direct mutagenic effects and unscheduled proliferation, in part due to the metabolism of the endogenous estrogens, estrone (E1) and estradiol (E2) by cytochrome P450 (CYP) enzymes, with subsequent creation of more potent estrogens and electrophilic intermediates. It has been suggested that the 2-hydroxy estrogens are benign, the 2-methoxy estrogens may be anti-carcinogenic through detoxification of electrophilic intermediates, and the 4- and 16α-hydroxy estrogens may be carcinogenic (2, 3).

Estrogen metabolism is regulated by the amount of substrate available and the expression and activity of cytochrome P450 (CYP) enzymes. In phase I metabolism, E1 and E2 are converted by CYP 1A/1B/3A to the relatively inactive metabolites 2-hydroxyestrone (2OH-E1) and 2-hydroxyestradiol (2OH-E2), respectively. Alternatively, E1 and E2 may be metabolized by CYP 1A/3A to 4-hydroxyestrone (4OH-E1) and 4-hydroxyestradiol (4OH-E2), metabolites shown to initiate cancer by forming DNA adducts. E1 may also be metabolized to 16-α-hydroxyestrone (16αOH-E1), a metabolite shown to covalently bind the estrogen receptor, signaling sustained estrogen receptor-mediated proliferation that may promote tumor growth (3, 4). In phase II metabolism, most of the 2-hydroxy metabolites are conjugated by catechol-*O*-methyltransferase (COMT) to 2-methoxyestradiol (2-ME2), a metabolite shown to inhibit carcinogenesis by inducing apoptosis and suppressing proliferation (5).

Most of the interest in estrogen metabolism and cancer has been in relation to breast cancer risk. Numerous studies have shown an inverse relationship between the ratio of urinary 2-hydroxy estrogens to 16α-hydroxyestrone (2:16 OH-E1) and breast cancer risk (6-13), although a few studies have not shown a significant association (14-16), and one study found an association in premenopausal but not postmenopausal women (17). Although only one prostate cancer case-control study has been reported in men, results were similar, with a trend toward lower 16αOH-E1 excretion, significantly higher 2OH-E1 excretion, and a significantly higher 2:16 OH-E1 ratio in controls than cases (18). These data are consistent with a pilot study that reported an inverse relationship between 2OH-E1 excretion and serum prostate specific antigen (PSA), a marker of prostate cancer (19).

In epidemiological studies, soy intake has been associated with decreased prostate cancer risk (20), but the mechanism is unknown and no studies have reported the effects of soy supplementation on urinary estrogen metabolism in men. In women, soy consumption has been shown to increase 2OH-E1 excretion (21-24), decrease 16αOH-E1 excretion (25), and increase the urinary 2:16 OH-E1 ratio (21, 22, 24, 25). One study reported an increased urinary 2:16 OH-E1 ratio only in women who metabolized the soy isoflavone daidzein to equol (24).

The aim of this study was to assess the effects of 6-month soy protein isolate consumption on urinary estrogen metabolites in men at high risk of prostate cancer. The effects of an isoflavone-rich soy protein isolate were compared to those of an isoflavone-poor soy protein isolate in order to elucidate whether isoflavones are the soy components responsible for altered estrogen metabolism. The underlying hypothesis was that isoflavone-rich soy protein isolate consumption would increase urinary E2 and

E1, 2OH-E1, 2-ME2 and the 2:16 OH-E1 ratio, and decrease-16αOH-E1, 4OH-E1, and 4OH-E2 excretion.

MATERIALS AND METHODS

The study population, design, and treatment have been discussed previously in detail (26). All 58 participants were recruited by urologic physicians at the Minneapolis Veteran's Administration Medical Center. The subjects were men between the ages of 50 and 85 years who recently underwent a prostate biopsy. Men were excluded from the trial if they were morbidly obese (BMI > 40 kg/m^2), had prostate cancer that required medical treatment, had chronic prostatitis, consumed more than fourteen alcoholic drinks per week, were allergic to soy or milk, used antibiotics frequently, or were on medically-prescribed protein restricted diets. All subjects provided written informed consent for participation in the trial, which was approved by the University of Minnesota Institutional Review Board: Human Subjects Committee, the Minneapolis Veterans Affairs Institutional Review Board, and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board.

The subjects were randomly assigned to consume one of three protein isolates for 6 months: 1) soy protein isolate (SPI+) containing 107 $\pm$ 5.0 mg isoflavones /d; 2) alcohol-extracted soy protein isolate (SPI-) containing < 6 $\pm$ 0.7 mg isoflavones /d; or 3) milk protein isolate (MPI) containing 0 mg isoflavones /d (The Solae Company; St. Louis, MO). The protein isolates were taken in divided doses twice daily, contributing a total of 40 g of protein and 200-400 kcals to the subjects' habitual diets each day. The mean distribution of isoflavones was 53% genistein, 35% daidzein, and 11% glycitein in SPI+, and 57% genistein, 20% daidzein, and 23% glycitein in SPI-, as analyzed by Dr. Pat

Murphy, Department of Food Science and Human Nutrition, Iowa State University. Participants recorded the time of consumption in study calendars, and compliance was assessed by self-report as detailed previously (26). To prevent any other soy isoflavone consumption, subjects were given a detailed list of soy-containing products to avoid. The men collected 24-hr urine samples one day prior to each of three clinic visits at 0, 3, and 6 months. The urine was collected in opaque plastic containers containing one gram of ascorbic acid per liter, then was preserved with 0.1% sodium azide, and aliquots were stored at –20°C until analysis. Urinary creatinine was measured by dry slide chemistry with a VITROS® Clinical Chemistry Analyzer (Ortho-Clinical Diagnostics, Raritan, NJ) and equol concentration was determined by high performance liquid chromatography and mass spectrometry (HPLC-MS) as previously described (27). For equol concentrations, the intra-assay coefficient of variation was 8.2%, and the inter-assay coefficient of variation was 12.5%.

Estrogen metabolites were measured by gas chromatography-mass spectrometry (GC-MS) using the method described below, modified from previously described methods (25, 28). Urine samples were thawed at room temperature, thoroughly mixed by vortex to ensure homogeneity and centrifuged at 5° C. for 5 min. Duplicate 10 ml aliquots of urine were added to clean, silanized 30 ml screw-top test tubes. Deuterated standards (C/D/N Isotopes, Pointe-Claire, Que, Canada) of all estrogen metabolites assayed were added to the urine, and an equal volume (10 ml) of ethoximation solution was added to the test tubes, thoroughly mixed by vortex and inversion and incubated overnight at room temperature (ca. 20-25° C).

The following day, the ethoximated samples were applied to Bond Elute LRC C-18 columns (Varian, Inc. Lake Forest, CA; 500 mg/ column). The C-18 columns had been pre-conditioned with a) 5 ml methanol and b) 10 ml of deionized-distilled (DD) water immediately prior to sample introduction. Columns were then washed with 5 ml of 0.15M acetate buffer, pH 3.0. Samples were eluted into a clean, silanized test tube with 3.0 ml of methanol, and then evaporated to dryness under nitrogen. The dry samples were hydrolyzed by dissolving in 5ml of a solution containing: 25 mg ascorbic acid, 50 µl β-glucuronidase (Sigma # G-7770, crude extract from *Helix pomatia)* in 0.15 M acetate buffer, pH 4.1 and incubated overnight at 37° C.

The following day, the hydrolyzed samples were applied to C-18 columns (conditioned as above), washed with 5 ml of DD water, and eluted into clean, silanized test tubes with 4.0 ml of methanol. Samples were evaporated to dryness under nitrogen, and derivized to their trimethyl silyl components with 200 µl of a 15% MSTFA+ TMCS solution in acetonitrile. (MSTFA+ 1% TMCS, Pierce Biotechnology Inc., Rockford, IL, prod #48915). Chromatographic analysis was performed on an HP 5890 Series II gas chromatograph equipped with an HP-1MS 15 m column (0.25 mm I.D., 0.25um film thickness) interfaced to an HP 5970 mass selective detector. Instrumental programmed control and quantitative analysis was performed using HP Chemstation software. All samples from a given subject were analyzed in the same batch, and an equal number of subjects from each group were included in each batch. Intra-assay coefficients of variation were between 3.5% and 6.4%, and inter-assay coefficients of variation were between 4.3% and 13.0%. Detection limits were 1.0 ng/mL for all estrogen metabolites except 2OH-E2 and 2OH-E1, which had detection limits of 0.50 ng/mL. 4-hydroxyestradiol, 4-

hydroxyestrone, 4-methoxyestradiol, and 4-methoxyestrone were undetectable in all subjects.

Subject retention. Subject accrual has been described previously in detail (26), with some variation described below. One subject that consented to the Soy and Prostate Cancer Prevention (SoyCaP) trial refused to collect his urine, and two other subjects were excluded from analysis due to missing baseline urine collections. Four subjects did not collect urine at all three time points [no mid-point urine (n = 1), no final urine (n = 3)]. Thus, 55 participants were evaluated at baseline, 54 were evaluated at 3 months, and 52 were evaluated at 6 months. One subject did not consume the treatment powder for 3 days prior to his 6 month appointment as a result of illness, so his data were excluded from the 6 month equol excretion analysis.

Statistical analysis. Analysis of covariance (SAS Proc GLM) was used to compare group means adjusted by their baseline values (29). For 16αOH-E1, the model included a bodyweight by baseline metabolite interaction. In addition, we carried out pre-planned pairwise comparisons as dictated by the study hypotheses. Paired t-tests were used to test for significant within-group changes. Skewed data were log transformed before analysis, and results are reported as geometric means and 95% confidence intervals. Data were analyzed both as nmol/d and nmol/mg creatinine, and since there were no differences, data are expressed as nmol/d. Statistical significance was defined as $P < 0.05$.

RESULTS

Baseline. All three groups (SPI+, SPI-, and MPI) had similar anthropometrics, cancer status, and dietary intake **(TABLE 1)**. The mean age of the men was 68 y, and mean

body mass index was 30 kg/m^2. The only significant difference among groups at baseline was that the SPI- group consumed more protein, but not at 3 and 6 months (26). Baseline urinary estrogen metabolites were similar except that the SPI+ group had higher 2-ME2 than the MPI group **(TABLE 2)**.

Urinary Estrogen Metabolites. (TABLE 2) In both soy groups, E2 concentrations were significantly increased from baseline at 3 and 6 months, and were significantly higher than the MPI group at both time points. A similar pattern was seen with respect to E1 concentrations at 6 months. There were no differences in 2OH-E1 concentrations over 6 months, but at 6 months 2OH-E2 concentration decreased significantly from baseline in the MPI group, and was therefore significantly lower than both soy groups. Both soy groups showed higher 16αOH-E1 concentrations than the MPI group at 3 months, but this disappeared at 6 months. The 2:16 OH-E1 ratio was significantly higher in the SPI+ group than the MPI group at 6 months.

Equol-excretor status. Equol excretor status was determined only in the SPI+ group, which received sufficient daidzein for equol production. There were 5 excretors and 14 non-excretors. However, only 2 excretors remained at 6 months, because one dropped out of the study after 3 months, data were excluded from another subject as discussed above, and one apparently changed excretor status. Therefore, only the 3 month data are reported. Baseline anthropometrics, cancer status, and dietary intake between excretors and non-excretors did not differ (26). At baseline, the equol excretors tended to have higher 2:16 OH-E1 concentrations than non-excretors (P = 0.06) **(TABLE 3)**. All measured estrogen metabolites were the same between equol excretors and non-excretors after 3 months of SPI+ consumption.

DISCUSSION

The primary objective of this study was to evaluate the effects of soy protein isolate consumption on the urinary estrogen profile in men at risk for developing advanced prostate cancer. Consumption of soy protein isolate, regardless of isoflavone content, increased urinary excretion of E2 and tended to increase excretion of E1. These results are similar to those from studies in postmenopausal women (23), and may be clinically relevant to prostate cancer prevention. Higher urinary excretion of E2 has been observed in prostate cancer controls compared to cases (30), and a high E2 concentration in the blood has been associated with decreased prostate cancer risk (31-33). Estrogens or estrogen analogs have been prescribed for decades to prostate cancer patients in order to decrease androgen production through negative feedback on the hypothalamic-pituitary-gonadal system. Our results suggest that increased E2 concentration may be one mechanism by which soy supplementation reduces the risk of prostate cancer. Our observation that this effect occurred regardless of isoflavone content of the soy protein isolate, suggests that isoflavones may not be the only hormonally-active compound in soy. Alternatively, it is possible that the small dose of isoflavones in the SPI- was sufficient to elicit a hormonal effect.

We previously showed that SPI- consumption, but not SPI+, increased serum E2 and E1 concentrations in this population (26). Given that our subjects did not have kidney disease and that hormone concentrations fluctuate throughout the day, it is likely that 24-hour urinary excretion is a more accurate reflection of estrogen synthesis than is circulating concentration measured at one time point. In addition, the GC-MS analytical

method used for urinary estrogen metabolites is considered more accurate than the radioimmunoassay method used for serum hormone analysis (34).

Urinary 2OH-E2 excretion decreased in the MPI group, but not in the soy groups, possibly due to higher E2 concentrations in the soy groups providing more substrate for the 2-hydroxy pathway than the control group. It has been suggested that soy consumption alters the enzymes involved in the formation of 2-hydroxy metabolites, including CYP 1A/3A (21, 35), although the data are somewhat inconsistent (35, 36).

Both soy groups had significantly higher urinary 16αOH-E1 excretion the MPI group at 3 months. These results are consistent with one study in postmenopausal women in which consumption of soy protein isolate containing 44 mg isoflavones/d tended to increase urinary excretion of 16αOH-E1 after a 6-week intervention (24). On the other hand, postmenopausal women consuming soy protein isolate containing 132 mg isoflavones/d for 3 months (23) and premenopausal women consuming soy protein isolate containing 129 mg isoflavones/d for 3 months (25) both had decreased urinary 16αOH-E1 excretion. Others have reported no effects of soy protein consumption on urinary 16αOH-E1 excretion in women (21, 37, 38).

Most importantly, this is the first study to show that soy protein isolate consumption alters the urinary ratio of 2:16 OH-E1 in men. The 2:16 OH-E1 ratio was higher in the SPI+ group than in the MPI group at 6 months, consistent with data from soy intervention studies performed in women (21, 22, 24, 25). An increased 2:16 OH-E1 ratio has been associated with a reduced risk of breast cancer in numerous studies (6-13, 39), but only one study has been published evaluating the relationship between the 2:16 OH-E1 ratio and prostate

cancer risk (18). This study showed that an increased 2:16 OH-E1 ratio was associated with a reduced risk of prostate cancer (18).

Within the SPI+ group, equol excretors tended to have a higher 2:16 OH-E1 ratio than non-excretors at baseline. This finding is consistent with data suggesting that there may be beneficial differences between equol excretors and non-excretors unrelated to the biological activity of equol itself (22, 40, 41). Our observation of no difference in the effects of soy consumption by equol excretor status is similar to previous reports in premenopausal and postmenopausal women (23, 25), although a few studies in women have reported an association between urinary equol excretion and a higher 2:16 OH-E1 ratio (22, 24, 42). Our analysis was likely limited by the small sample size, and the results are preliminary and should be interpreted with caution.

To our knowledge, this is the first study to report the full profile of urinary estrogen metabolites in men at high risk of developing prostate cancer, and the first to report the effects of soy consumption on estrogen metabolite excretion in men. Consumption of soy protein isolate, regardless of isoflavone content, increased estrogen excretion, and SPI+ consumption, but not SPI-, increased the 2:16 OH-E1 ratio. Given that increased estrogens and 2:16 OH-E1 ratio have been associated with lower prostate cancer risk, our data suggest that effects on endogenous estrogen synthesis and metabolism may contribute to the prostate cancer preventive effects of soy consumption.

ACKNOWLEDGEMENTS

This research was funded by the United States Army Department of Defense Prostate Cancer Research Program under awards DAMD 17-02-1-0101 (MSK) and W81XWH-06-1-0075 (JHR). Views and opinions of, and endorsements by the author do not reflect

those of the US Army or the Department of Defense. The protein isolates were generously donated by The Solae Company, St. Louis, MO. The authors would like to thank Tony Vosooney and Mike Wachter for performing the analytical work, and Lori Sorensen, Nicole Nelson, and Mary McMullen for assistance with clinic visits and data entry. We are also quite grateful to the study participants for their time and dedication.

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TABLE 1: Baseline characteristics of subjects [†]

	SPI+ n = 19	SPI- n = 19	MPI n = 17
Age (y)	68 ± 8	68 ± 6	69 ± 6
Body wt (kg)	91 ± 16 *ab*	89 ± 12 *a*	98 ± 15 *b*
Height (cm)	174 ± 7	174 ± 8	177 ± 8
BMI (kg/m^2)	30 ± 5	30 ± 4	32 ± 6
Prostate Cancer Markers [§]			
PIN *n* (%)	17 (89)	17 (89)	14 (82)
ASAP *n* (%)	3 (16)	6 (32)	3 (18)
CaP *n* (%)	2 (11)	1 (5)	2 (12)
Dietary Intake			
Energy intake (kcal/d)	2088 ± 590	2335 ± 590	2092 ± 530
Protein (g/d)	80 ± 15 *a*	103 ± 24 *b*	81 ± 25 *a*
Carbohydrate (g/d)	254 ± 109	270 ± 116	239 ± 59
Fat (g/d)	83 ± 31	96 ± 29	89 ± 24
Dietary fiber (g/d)	17 ± 9	18 ± 7	17 ± 5

[†] All values are means ± SD except prostate cancer markers which are n (%).

[§] Prostate cancer markers PIN, ASAP, and CaP are not mutually exclusive.

[ab] Means in a row without a common letter differ ($P < 0.05$).

Abbreviations: SPI+, isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI-, alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI, milk protein isolate (40 g milk protein);

PIN, prostatic intraepithelial neoplasia; ASAP, atypical small acini suspicious for prostatic adenocarcinoma; CaP, prostate cancer

TABLE 2: Urinary estrogen metabolites (nmol/d) [†]

	SPI+ *n* = 20 [§]	**SPI-** *n* = 20 [§]	**MPI** *n* = 18 [§]
Estradiol (E2)			
Baseline	51 (34, 74)	42 (27, 63)	49 (29, 81)
3 Mo	* 94 (65, 135) *a*	* 76 (53, 111) *a*	44 (30, 66) *b*
6 Mo	* 91 (63, 132) *a*	* 90 (63, 129) *a*	50 (34, 72) *b*
Estrone (E1)			
Baseline	20 (15, 28)	18 (13, 26)	25 (20, 31)
3 Mo	26 (20, 34)	21 (16, 28)	22 (16, 29)
6 Mo	* 37 (28, 49) *a*	* 27 (20, 35) *b*	23 (17, 30) *b*
2-methoxyestradiol (2-ME2)			
Baseline	57 (39, 84) *a*	42 (31, 56) *ab*	31 (21, 47) *b*
3 Mo	39 (26, 58)	42 (28, 62)	37 (24, 57)
6 Mo	26 (18, 38)	36 (25, 52)	34 (23, 50)
2-methoxyestrone (2-ME1)			
Baseline	9.1 (7, 12)	9.5 (6, 15)	8.1 (6, 12)
3 Mo	7.6 (5, 12)	9.5 (6, 15)	8.9 (6, 14)
6 Mo	9.8 (7, 15)	9.2 (6, 14)	7.9 (5, 12)

Estriol (E3)			
Baseline	55 (42, 71)	28 (14, 57)	47 (29, 78)
3 Mo	28 (19, 42) *a*	56 (38, 84) *b*	41 (27, 63) *ab*
6 Mo	31 (19, 49)	45 (28, 72)	42 (26, 67)
2-hydroxyestradiol (2OH-E2)			
Baseline	7.3 (5, 12)	4.8 (3, 8)	5.9 (3, 11)
3 Mo	7.2 (5, 11)	6.9 (4, 11)	5.6 (4, 9)
6 Mo	5.6 (4, 8) *a*	8.3 (6, 12) *a*	* 3.0 (2, 4) *b*
2-hydroxyestrone (2OH-E1)			
Baseline	20 (13, 30)	21 (13, 33)	26 (19, 36)
3 Mo	21 (14, 30)	23 (16, 34)	26 (17, 38)
6 Mo	29 (21, 41)	25 (18, 35)	21 (15, 30)
16α-hydroxyestrone (16αOH-E1)			
Baseline	6.0 (4, 9)	5.7 (4, 9)	6.9 (5, 10)
3 Mo	7.4 (5, 11) *a*	7.9 (5, 11) *a*	4.5 (3, 7) *b*
6 Mo	6.0 (4, 9)	7.3 (5, 11)	6.6 (5, 10)
2:16 OH-E1 ratio (mean ± SE)			
Baseline	7.8 ± 1	7.5 ± 1	7.8 ± 2
3 Mo	8.0 ± 2	8.5 ± 2	10.4 ± 2
6 Mo	11.3 ± 2 *a*	8.2 ± 2 *ab*	5.1 ± 2 *b*

[†] Baseline data are unadjusted geometric means ± 95% CI except 2:16 OH-E1 ratio data which are means ± SE. All other data are least-squares geometric means adjusted for baseline measurement ± 95% CI, except 16αOH-E1 which

is additionally adjusted for baseline weight and 2:16 OH-E1 ratio data which are least-squares means ± SE and were analyzed on the original scale.

[§] Sample sizes listed at column headings are for all time points except the 3 mo MPI (n = 16), 6 mo SPI+ (n = 17), and 6 mo SPI- (n = 18) timepoints.

[ab] Means in a row without a common letter differ ($P < 0.05$).

*Significant within-group change from baseline ($p < 0.05$).

Abbreviations: SPI+, isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI-, alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI, milk protein isolate (40 g milk protein); 2:16 OH-E1 ratio, [(2OH-E1 + 2OH-E2) /16αOH-E1]

TABLE 3: Urinary estrogen metabolites between equol excretors and non-excretors (nmol/d) [†]

	Excretors *n = 5*	**Non-excretors** *n = 14*
Estradiol (E2)		
Baseline	44 (22, 84)	53 (32, 88)
3 Mo	78 (30, 206)	107 (60, 189)
Estrone (E1)		
Baseline	20 (10, 40)	20 (13, 31)
3 Mo	21 (11, 41)	27 (18, 40)
2-methoxyestradiol (2-ME2)		
Baseline	58 (24, 139)	57 (35, 93)
3 Mo	31 (12, 79)	47 (27, 82)
2-methoxyestrone (2-ME1)		
Baseline	9.7 (5, 18)	8.8 (6, 13)
3 Mo	9.8 (5, 21)	7.1 (5, 11)
Estriol (E3)		
Baseline	70 (40, 125)	50 (36, 68)
3 Mo	20 (6, 69)	39 (19, 79)

2-hydroxyestradiol (2OH-E2)		
Baseline	4.9 (3, 50)	7.6 (5, 11)
3 MO	4.9 (2, 13) [§]	9.2 (5, 17)
2-hydroxyestrone (2OH-E1)		
Baseline	32 (11, 96)	17 (10, 27)
3 Mo	17 (5, 51)	21 (11, 41)
16α-hydroxyestrone (16αOH-E1)		
Baseline	6.3 (3, 15)	5.9 (4, 9)
3 Mo	8.8 (4, 20)	6.5 (4, 10)
2:16 OH-E1 ratio (mean ± SE)		
Baseline	12.0 ± 2	6.4 ± 1
3 Mo	6.8 ± 3	8.6 ± 2

[†] Baseline data are unadjusted means ± 95% CI except 2:16 OH-E1 ratios which are means ± SE. Data from 3 months are least-squares means adjusted for baseline measurement ± SE, except 16αOH-E1which is additionally adjusted for interaction between treatment and baseline, and 2:16 OH-E1 ratio data which are least-squares means ± SE and were analyzed on the original scale.

[§] $n = 13$

[ab] Means in a row without a common letter differ ($P < 0.05$).

* Significant within-group change from baseline ($p < 0.05$).

Abbreviations: 2:16 OH-E1 ratio, [(2OH-E1 + 2OH-E2) /16αOH-E1]

Appendix C

Effects of soy protein isolate consumption on prostate cancer biomarkers in men at high risk of prostate cancer: results from the SoyCap trial

J. M. HAMILTON-REEVES[1], S. A. REBELLO[1], W. THOMAS[2], M. S. KURZER[1*], and J. W. SLATON[3]

[1]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108, USA

[2]Division of Biostatistics in the School of Public Health, University of Minnesota, Minneapolis, MN 55455, USA

[3] Department of Urologic Surgery, University of Minnesota, Minneapolis, MN 55455 and Department of Urology Veterans Administration Medical Center, Minneapolis, MN 55417, USA

[Running title: SOY AND TUMOR MARKERS]

ABSTRACT WORD COUNT: 306

MANUSCRIPT WORD COUNT: 3117 (*including section headers*)

[*] Corresponding author: Dr. Joel Slaton, Department of Urologic Surgery, University of Minnesota, Minneapolis, MN 55455 and Department of Urology Veterans Administration Medical Center, Minneapolis, MN 55417, USA, Phone: (612) 725-3460; Fax: (612) 625-5272; E-mail: slato001@umn.edu.

[†] Work was performed at the department of Urology Veterans Administration Medical Center, Minneapolis, MN 55417, USA

[§] The Soy and Prostate Cancer Prevention (SoyCaP) trial was supported by grant DAMD 17-02-1-0101 (MSK) and W81XWH-06-1-0075 (JHR) from the United States Army Department of Defense Prostate Cancer Research Program. The protein isolates were donated by The Solae Company, St. Louis, MO. Neither sponsor was involved in writing this report.

Abstract

Background. Epidemiological studies suggest that diets high in soy are associated with a decreased risk for prostate cancer. In rodents, phytoestrogenic soy isoflavones have been shown to alter prostate cancer biomarkers including those associated with decreased cell proliferation, increased apoptosis and downregulated epidermal growth factor receptor (EGFr).

Methods. Fifty-eight men at high risk for prostate cancer were randomly assigned to consume one of three soy protein isolates containing 40 g protein: 1) soy protein isolate (SPI+, 107 mg isoflavones/d); 2) alcohol-washed soy protein isolate (SPI-, < 6 mg isoflavones/d); or 3) milk protein isolate (MPI, 0 mg isoflavones/d). Expression of cancer biomarkers (PCNA, EGFr, Bax, and Bcl-2) were assessed by immunohistochemical histological score in baseline and ending prostate biopsy cores. Serum samples collected at 0, 3, and 6 months were analyzed by chemiluminescence immunoassay for total and free prostate specific antigen (PSA).

Results. Consumption of SPI+ did not alter any of the prostate cancer tumor markers analyzed. Bax expression significantly decreased from baseline in the SPI- group, resulting in significantly lower Bax expression than the MPI group. PCNA expression also significantly decreased from baseline in the SPI- group but this was not significantly different from the other two groups. Total PSA, free PSA, PSA percent, and PSA density did not differ among the groups at 3 or 6 months. A trend toward a lower rate of prostate cancer development in men in the soy groups compared to the milk group was observed (*P=0.09*).

Conclusions. These data suggest that 6 month consumption of isoflavone-rich soy protein isolate does not alter prostate tissue biomarkers of prostate cancer risk in high risk men, although consumption of isoflavone-poor alcohol-washed soy protein isolate exerted mixed effects. After 6 months, men in the soy groups were less likely to progress to a malignant diagnosis than the men in the milk group.

KEY WORDS: isoflavones, apoptosis, proliferation, PCNA, Bax

Introduction

Men with biopsy-proven low grade cancer, or nonmalignant preneoplastic lesions such as atypical small acini suspicious for prostatic adenocarcinoma (ASAP) or high-grade prostatic intraepithelial neoplasia (HGPIN) must weigh the risks and benefits of therapy and are often counseled not to undergo conventional treatments. These men would be ideal candidates for a non-toxic dietary supplement with proven efficacy for reversing or retarding these early prostate tissue lesions.

Phytoestrogenic soy isoflavones have been shown to exert prostate cancer preventive effects, and soy consumption has been associated with decreased prostate cancer risk in epidemiological studies (1). Isoflavone supplementation has been shown to suppress serum prostate specific antigen (PSA), a biomarker associated with prostate cancer progression. In men with prostate cancer, soy food interventions have significantly decreased mean total serum PSA compared to controls (2, 3), although several studies have not shown statistically significant effects of soy or isoflavone consumption on total PSA (4-15). Since total PSA is a non-specific biomarker for prostate cancer, clinicians often evaluate the free to total PSA percent to differentiate between cancer and benign conditions (16). The lower the value of free PSA percent, the greater the probability that elevated PSA represents cancer and not benign prostatic hyperplasia. In men with PSA concentrations between 4 and 10 ng/mL and a free PSA percent below 10 percent, risk of cancer is 56 percent, compared to men with a free PSA percent above 25 percent, whose risk of cancer is only 8 percent (17). Only two studies to date have evaluated the effects of soy or isoflavone consumption on free PSA percent. Dalais et al (3) reported that soy grits increased free PSA percent, but Kranse et al did not observe a change in free PSA percent

with isoflavone supplementation (10).

It has been suggested that intra-prostatic expression of antigens related to carcinogenesis may be useful molecular biomarkers in dietary intervention studies (18). Soy isoflavone interventions in various models have decreased cell proliferation, downregulated the epidermal growth factor receptor (EGFr), and increased programmed cell death, or apoptosis. Soy has suppressed cell proliferation as detected by proliferating cell nuclear antigen (PCNA) staining in rodents dosed with either soy protein concentrate (19) or physiologic concentrations of the isoflavone, genistein (20). Physiological doses of dietary genistein have down-regulated EGFr mRNA expression during the early phase of prostate cancer development in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (20, 21). Increased prostate tissue apoptosis has been shown in prostatectomy specimens obtained from patients treated with isoflavones derived from red clover when compared to historically matched controls (8), and soy protein concentrate has increased apoptotic index in the immune compromised mouse model (19). Genistein has increased apoptosis in vitro as detected by the pro-apoptotic signaling protein, Bcl-2-associated X protein (Bax) (22). Comparing Bax to the anti-apoptotic signaling protein, B-cell non-Hodgkin lymphoma-2 (Bcl-2) has been validated to indicate apoptosis status in prostate biopsy specimens (23, 24).

The aim of this study was to assess the effects of soy protein isolate consumption on prostate cancer biomarkers in men at high risk of prostate cancer. A randomized placebo-controlled trial was performed in 58 men who consumed either isoflavone-rich soy protein isolate, isoflavone-poor soy protein isolate or milk protein isolate for 6 months. The purpose of this study was to evaluate the efficacy of a soy intervention at

the beginning of prostate carcinogenesis, and to determine whether or not isoflavones are the responsible bioactive components of soy. The underlying hypothesis was that isoflavone-rich soy protein isolate consumption would increase Bax and decrease Bcl-2, EGFr, PCNA, and serum PSA.

Materials and Methods

The six-month randomized controlled trial was conducted at the Minneapolis Veteran's Administration Medical Center and was approved by the University of Minnesota Institutional Review Board: Human Subjects Committee, the Minneapolis Veterans Affairs Institutional Review Board, and the U.S. Army Medical Research and Materiel Command's Human Subjects Research Review Board. Subjects were recruited from a pool of patients who, due to their high-risk status, had already undergone a transrectal ultrasound and biopsy, and the biopsy results showed either preneoplastic lesions (*n* = 53) or low-grade prostate cancer with Gleason scores of 6 or below (*n* = 5). Subjects were considered high risk if they had high-grade prostatic intraepithelial neoplasia (PIN) (*n* = 50) and/or ASAP (atypical small acinar proliferation) (*n* = 4). The 5 patients with low-grade prostate cancer were not on any other prostate cancer treatments. Urologists invited patients to participate in the study at their post-biopsy clinic visit, and the patients' medical records were reviewed by a research nurse to determine eligibility. Patients were not allowed to participate if they were morbidly obese (BMI > 40 kg/m^2), had prostate cancer that required medical treatment, had chronic prostatitis, consumed more than fourteen alcoholic drinks per week, were allergic to soy or milk, used antibiotics frequently, or were on medically-prescribed protein restricted diets.

All 58 subjects supplemented their habitual diets twice daily with one of three study protein isolates: 1) soy protein isolate (SPI+); 2) alcohol-extracted soy protein isolate (SPI-); or 3) milk protein isolate (MPI) (The Solae Company; St. Louis, MO). The protein isolates provided 40 g protein/d and 200-400 kcal/d. The isoflavone content (analyzed by Dr. Pat Murphy, Department of Food Science and Human Nutrition, Iowa State University) was 107 $\pm$ 5.0 mg/d for the SPI+; < 6 $\pm$ 0.7 mg/d for the SPI-; and 0 mg/d for the MPI (mean $\pm$ SD), expressed as aglycone equivalents. The mean distribution of isoflavones was 53% genistein, 35% daidzein, and 11% glycitein in SPI+, and 57% genistein, 20% daidzein, and 23% glycitein in SPI-. Compliance was assessed by self-report as detailed previously (Hamilton-Reeves, in press). To minimize isoflavone consumption from other sources, subjects were given a detailed list of soy-containing products to avoid.

Subject retention has been previously described in detail (Hamilton-Reeves et al, in press). Data from 58 participants were included in the serum PSA analysis (*n* = 58), and data from 44 subjects were included in the antigen expression analysis. Fewer participants were eligible for antigen expression analysis because 7 subjects did not undergo the final prostate biopsy [liver cancer (*n* = 1), heart complication (*n* = 1), not clinically indicated (*n* = 1), opted out of procedure (*n* = 1), early withdrawal from study (*n* = 3)], and 7 subjects had insufficient biopsy tissue at either baseline or study-end for the analyses.

Serum collection and analysis. Participants reported for clinic visits at 0, 3, and 6 months. Fasting blood was drawn in the morning. Serum was separated and frozen at –70°C until analysis. Serum PSA was measured in one batch at the Minneapolis

Veteran's Administration Hospital by the Architect total PSA chemiluminescence microparticle immunoassay (Architect ci8200, Abbott Laboratories, Chicago, IL). Intra-assay variability was 2.5%. Free PSA was measured in one batch at Associated Regional and University Pathologists (ARUP) Laboratories by the Roche Modular E170 free PSA electrochemiluminescent immunoassay. Intra-assay variability was 7.1%.

Tissue collection and analysis. Prostate cores were obtained before the initial screening and obtained again after the 6-month dietary intervention. Biopsy cores were fixed in formalin and paraffin embedded. The paraffin-embedded blocks were sectioned onto slides, and the slides were evaluated and diagnosed by the pathologist at the Minneapolis Veteran's Administration Hospital. After diagnosis, slides were obtained from pathology to perform immunohistochemistry for PCNA, EGFr, Bax, and Bcl-2 expression. The tissue sections were deparaffinized, rehydrated in graded alcohol, and transferred to PBS (pH 7.3). Epitope retrieval was induced by pressure-cooking at 15 psi in citrate buffer with a pH of 6.0 for 10 minutes and submerged in quenching solution (3% H_2O_2 in 100% MeOH) for 5 minutes. After blocking (10% milk, 5% serum, and 1% bovine serum albumin), the samples were incubated overnight at 4° C with mouse monoclonal anti-PCNA antibody (555566; BD Biosciences, San Diego, CA; 1:500), mouse monoclonal anti-Bcl-2 antibody (551107; BD Biosciences, San Diego, CA; 1:500), or rabbit polyclonal anti-Bax antibody (554104; BD Biosciences, San Diego, CA; 1:1000). The samples for the EGFr assay were incubated at room temperature for 30 minutes with the mouse monoclonal anti-EGFr antibody (08-1205; Zymed, Invitrogen Corporation, Carlsbad, CA; RTU). After rinsing, the samples were incubated with the corresponding biotinylated secondary antibody. Vectastain Elite ABC kit (Vector

Laboratories, Burlingame, CA) and diaminobenzidine (DAB) were utilized to stain the expressed antigens brown. The slides were rinsed with water and counterstained with Harris' Modified Hematoxylin (Fisher Scientific, Pittsburgh, PA). After the slides were dehydrated, cleared, and mounted, the slides were photographed, and the digital images were optimized for scoring as described in our previous report (Hamilton-Reeves et al, in press). Positive and negative controls were run in each batch, and the images were scored using the immunohistochemical histological score (HSCORE) semiquantative method (25). The HSCORE is a sum of the percentage of epithelial cells weighted by their staining intensity above control. HSCORE = Σ PC (i + 1); where i is the intensity of staining with a value of 0 (absent), 1 (weak), 2 (moderate), or 3 (strong), PC is the percentage of stained epithelial cells for each intensity varying from 0 to 100%. The range of the HSCORE is a minimum of 1 and a maximum of 4. The immunostained slides were evaluated independently by two technicians blinded to each patient's medical history. There was good agreement between the two observers; the spearman correlation between them was 0.8. On average 5 intact glands (range: 3 – 8) were scored per subject slide. Due to poor staining or incomplete glands on tissue sections some slides were not scored, thus a few patients were excluded from analysis: PCNA (*n* = 3) and EGFr (*n* = 2).

Statistical Analysis. The data were tested for normality, and there were no major outliers in any treatment groups. Analysis of covariance (SAS Proc GLM) was used to compare group HSCORE means or differences among the group PSA means adjusted by their corresponding baseline values (SAS Institute Inc., *SAS® 9.1*, Cary, NC: SAS Institute Inc., 2003). In addition, pre-planned pairwise comparisons were carried out as dictated

by the study hypotheses. Due to small cell numbers (< 5), Fischer's exact test was used to compare cancer incidence rates among the groups at study end. Paired t-tests were used to test for significant within-group changes over time. $P < 0.05$ was considered statistically significant.

Results

Baseline. Anthropometrics, cancer status, and dietary intake did not differ between treatment groups, as described previously (Hamilton-Reeves, in press). The average age for all men was 68 y; the average body mass index was 30 kg/m ^{2}. At baseline, aggregate antigen expression HSCORES did not differ among the groups (TABLE 1). Similarly, there were no differences in baseline total or free PSA concentrations, prostate volume, or PSA density among the groups (TABLES 2 & 3).

Antigen Expression. After 6 months, Bax expression was lower in prostate biopsies in the SPI- group compared to the MPI group (pairwise comparison, $P = 0.03$) and approached a significant difference compared to the SPI+ group (pairwise comparison, $P = 0.10$) (TABLE 1). PCNA expression was decreased from baseline in the SPI- group, but baseline-adjusted PCNA expression was not significantly different from the other two groups. There were no effects of treatment or differences among the groups in Bcl-2, EGFr, Bax: Bcl-2 ratio, or Bax: PCNA ratio.

PSA and Prostate Volume. There were no effects of treatment or differences among the groups in total PSA, free PSA, or PSA percent (TABLE 2). Prostate volume at 6 months was increased in the SPI- group relative to the MPI group (pairwise comparison, P = 0.04), but PSA density (serum total PSA/prostate volume) was not different among the groups (TABLE 3).

Cancer Incidence. Of the 55 men without cancer at baseline, 54 underwent 12 core biopsies that were evaluated by the pathologist (one subject was not clinically indicated to undergo the final prostate biopsy). After the 6 month intervention, there was a trend toward fewer men progressing to cancer in the soy groups compared to the MPI group (P = 0.09). Prostate cancer risk was 38% (n = 6/10) in the MPI group, versus 17% (n = 3/15) in the SPI+ group and 16% (n = 3/16) in the SPI- group.

Disscussion

This study evaluated the effects of soy protein isolate on prostate tissue antigen expression levels, serum total and free PSA, prostate volume, and PSA density. Consumption of isoflavone-rich soy protein isolate had no effects on any of the prostate cancer tumor markers analyzed. However, in the post-intervention biopsy tissue from the men consuming alcohol-extracted soy protein isolate, we observed lower Bax expression levels (reflecting decreased apoptosis) compared to those consuming MPI and decreased PCNA expression levels (reflecting decreased proliferation) compared to baseline values. Despite these seemingly contradictory effects, there was a trend toward decreased risk of cancer in the soy groups compared to the MPI group.

The lack of effect of SPI+ consumption on total PSA concentrations is consistent with several soy or isoflavone intervention studies in which no change in total PSA was observed (4-13), but inconsistent with a few reports of significant reductions (2, 3) or trends toward reductions (14, 15) in total PSA concentrations. Since a nearly significant difference in prostate volume was observed, we also evaluated total PSA standardized to prostate size (PSA density). Neither PSA density nor free PSA concentrations were different among the groups, consistent with all studies to date (3, 4, 10, 12, 14). We also

found no effect of treatment on free PSA percent, consistent with Kranse et al (10), but inconsistent with Dalais et al who reported increased free PSA percent (3).

Consumption of SPI+ did not affect the expression of the apoptotic cancer biomarkers, Bax and Bcl-2, analyzed in baseline and ending prostate biopsy cores. The lack of effect on apoptotic markers is in contrast to in vitro data showing increased Bax when LNCaP cells were exposed to 100 μM genistein (22), and one study reporting higher apoptotic index in prostate specimens obtained from men who consumed isoflavone extract compared to historically matched controls (8). The disparity between the results of this last study (8) and our results could be explained by different treatment regimens (red clover vs SPI), control groups (historically matched vs placebo controlled), and analytical methods for apoptosis (apoptotic index vs specific signaling proteins, ie Bax and Bcl-2). Although comparing Bax to the anti-apoptotic signaling protein Bcl-2 has been validated to indicate apoptosis status in prostate cancer biopsy specimens (23, 24), most of our subjects did not have prostate cancer. Bcl-2 was scored only in the luminal layer, and consistent with the literature we found that benign glands had minimal to absent staining of Bcl-2 in these cells (26). Given the small range in Bcl-2 HSCORE in our study, larger tissue sections and more subjects would need to be evaluated for improved reliability of the Bax to Bcl-2 ratio within precancerous lesions. Thus, utilizing biopsy cores from preneoplastic prostate glands for this endpoint was a limitation of our study design.

Although consumption of SPI+ did not influence Bax, consumption of SPI- significantly decreased Bax from baseline such that at 6 months it was lower than the other two groups. These findings are consistent with the hypothesis that isoflavones

increase Bax, and suggest that a different constituent of SPI+ decreases Bax, resulting in a neutral effect when they are present together and a reduction of Bax when isoflavones are removed (27-29).

Consumption of SPI+ did not alter PCNA, whereas consumption of SPI- decreased PCNA from baseline, although there were no differences among the groups at 6 months. These results are inconsistent with rodent studies showing that soy protein concentrate (19) or physiologic concentrations of genistein (20) suppressed PCNA staining. PCNA is an auxiliary protein of DNA polymerase that reaches maximal expression during the DNA replication phase (S phase) of the cell cycle. Therefore, abundant PCNA in the cell reflects DNA replication, and several studies have confirmed that PCNA index is directly correlated with prostate cancer progression (30-33).

We found no change in EGFr by either SPI+ or SPI- consumption, which is in contrast to animal studies in which dietary genistein down-regulated EGFr mRNA expression during the early phase of prostate cancer development in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model at physiologically plausible doses (20, 21). EGFr activates transcription through either the EGFr-Shc-SOS-Ras-Raf-ERK1/2 or the phosphatidylinositol-3'kinase-AKT pathways leading to cellular proliferation, angiogenesis, and apoptosis evasion. A limitation of our study is that we measured EGFr expressed, not phosphorylated or activated. Thus, further studies are needed to evaluate the effects of SPI consumption on the activation of the EGFr pathways.

Lastly, we observed a trend toward different rates at which patients progressed to a malignant diagnosis at study end. Malignancy was diagnosed two times less

frequently in the soy group than in the MPI group. Though interesting and relevant, this finding should be interpreted with caution, given that this short term study was not designed to investigate progression to cancer. In light of data associating soy consumption with decreased prostate cancer risk in epidemiological studies (1), and mechanistic evidence of hormonal changes in this population (Hamilton-Reeves, in press), further soy interventions designed with prostate cancer onset or progression as endpoints are warranted.

To our knowledge, this is the first randomized controlled study on the effects of a soy protein isolate intervention on prostate tissue biomarkers in men at high risk of developing prostate cancer. Consumption of isoflavone-rich soy protein isolate had no effects on any of the prostate cancer tumor markers analyzed. However, consumption of alcohol-washed (isoflavone-poor) soy protein isolate had mixed effects, decreasing pro-apoptotic Bax expression levels and decreasing proliferation as reflected in PCNA expression levels. These data suggest that there may be multiple constituents of soy protein isolate that exert varied effects on prostate cancer biomarkers. Importantly, we observed a trend toward a lower rate of prostate cancer development in men in the soy groups compared to the milk group. Further research should be conducted to determine whether soy delays the onset and progression of clinically significant prostate cancer, and to identify the responsible soy components.

Acknowledgements

This research was funded by the United States Army Department of Defense Prostate Cancer Research Program under award numbers DAMD 17-02-1-0101 and W81XWH-06-1-0075. Views and opinions of, and endorsements by the author do not reflect those

of the US Army or the Department of Defense. The protein treatment powders were generously donated by The Solae Company, St. Louis, MO. Immunohistochemistry was performed by Kenji Takamura. The authors would like to thank Kayla Vettling and Ellie Wiener for tissue scoring, and Lori Sorensen, Nicole Nelson, and Mary McMullen for assistance with clinic visits and data entry. We are also quite grateful to the study participants for their time and dedication.

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TABLE 1: Antigen expression (HSCORE) [1]			
	SPI+	**SPI-**	**MPI**
Bax [2]			
Baseline	1.38 ± 0.08	1.45 ± 0.07	1.35 ± 0.06
6 Mo	1.41 ± 0.06 *ab*	*1.27 ± 0.05 *a*	1.44 ± 0.06 *b*
PCNA [3]			
Baseline	1.61 ± 0.1	1.93 ± 0.1	1.86 ± 0.1
6 Mo	1.69 ± 0.1	*1.57 ± 0.1	1.81 ± 0.1
Bcl-2 [4]			
Baseline	1.11± 0.03	1.17 ± 0.07	1.09 ± 0.03
6 Mo	1.15 ± 0.04	1.15 ± 0.04	1.19 ± 0.04
EGFr [5]			
Baseline	1.34 ± 0.08	1.42 ± 0.10	1.39 ± 0.11
6 Mo	1.36 ± 0.06	1.37 ± 0.06	1.33 ± 0.06
Bax: Bcl-2 ratio [6]			
Baseline	1.25 ± 0.07	1.30 ± 0.10	1.23 ± 0.06
6 Mo	1.20 ± 0.05	1.14 ± 0.05	1.22 ± 0.05
Bax: PCNA ratio [7]			
Baseline	0.88 ± 0.05	0.76 ± 0.05	0.76 ± 0.05
6 Mo	0.89 ± 0.05	0.82 ± 0.05	0.84 ± 0.05

[1] Baseline data are unadjusted means ± SE. All other data are least-squares means adjusted for baseline measurement ± SE.

[2] n = 14 for SPI+, 16 for SPI-, and 14 for MPI.

[3] n = 14 for SPI+, 13 for SPI-, and 14 for MPI

[4] n = 15 for SPI+, 14 for SPI-, and 16 for MPI

[5] n = 15 for SPI+, 14 for SPI-, and 13 for MPI

[6] n = 14 for SPI+, 14 for SPI-, and 13 for MPI

[7] n = 13 for SPI+, 13 for SPI-, and 12 for MPI

[ab] Means in a row without a common letter differ ($P < 0.05$).

*Significant within-group change from baseline ($P < 0.05$).

Abbreviations: SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI = milk protein isolate (40 g milk protein)

TABLE 2: Serum PSA differences from baseline [1]

	SPI+ n = 20	SPI- n = 20	MPI n = 18
Total PSA (ng/mL)			
Baseline	5.4 ± 1	5.0 ± 1	5.1 ± 1
3 Mo Change	-0.8 ± 0.5	-0.8 ± 0.5	-0.6 ± 0.6
6 Mo Change	-0.5 ± 0.6	-0.8 ± 0.6	-0.2 ± 0.6
Free PSA (ng/mL)			
Baseline	0.9 ± 0.09	0.8 ± 0.1	0.9 ± 0.2
3 Mo Change	-0.09 ± 0.09	0.04 ± 0.09	-0.10 ± 0.1
6 Mo Change	-0.07 ± 0.07	-0.02 ± 0.07	-0.06 ± 0.07
PSA Percent			
Baseline	22 ± 2	19 ± 2	22 ± 2
3 Mo Change	-0.21 ± 1	0.67 ± 1	-0.74 ± 1
6 Mo Change	1.03 ± 1	1.18 ± 1	-0.22 ± 1

[1] Baseline data are unadjusted means ± SE. Differences are post-intervention minus baseline and are least-squares means adjusted for baseline measurement ± SE.

[2] Sample sizes listed at column headings are for all time points except 3 mo MPI (n = 17), 6 mo SPI+ (n = 18), and 6 mo SPI- (n = 19).

Abbreviations: PSA = prostate specific antigen; PSA Percent = free PSA/total PSA; SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI = milk protein isolate (40 g milk protein)

TABLE 3: Prostate volume and PSA density differences from baseline

	SPI+ n = 10	SPI- n = 13	MPI n = 15
Prostate Volume (cm^3)			
Baseline	52 ± 5	47 ± 5	54 ± 6
6 Mo Change	-4.3 ± 3 *ab*	1.6 ± 2 *a*	-5.5 ± 2 *b*
PSA Density (ng/mL/cc)			
Baseline	0.1 ± 0.03	0.09 ± 0.02	0.1 ± 0.02
6 Mo Change	0.0001 ± 0.01	-0.003 ± 0.01	-0.005 ± 0.01

[1] Baseline data are unadjusted means ± SE. Differences are post-intervention minus baseline and are least-squares means adjusted for baseline measurement ± SE.

[ab] Means in a row without a common letter differ ($P < 0.05$).

Abbreviations: PSA = prostate specific antigen; PSA density = total PSA/prostate volume; SPI+ = isoflavone-rich soy protein isolate (40 g soy protein, 107 mg isoflavones/d); SPI- = alcohol-extracted soy protein isolate (40 g soy protein, < 6 mg isoflavones/d); MPI = milk protein isolate (40 g milk protein)